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13. ABSTRACT *(Maximum 200 Words)*

We have studied the effects of various Vitamin D analogs alone and in combination against breast cancer cells. We have found the vitamin D analog EB1089 to be particularly potent against *in vivo* breast cancer cells (MCF-7), without causing hypercalcemia or other major side-effects. An additive effect was observed when a vitamin D analog and Taxol were administered together. EB1089 plus Taxol was the most active combination. Our studies have also demonstrated that a peroxisome proliferator activated receptor gamma (PPARγ) ligand (Troglitazone) has chemopreventive properties in breast cancer. The addition of a RXR-selective ligand appears to enhance this activity. Cyr61 is a gene that has been identified in other tissue types and is associated with angiogenesis and metastasis. We have identified prominent expression of the Cyr61 gene in selected breast cancer cell lines as well as in fresh breast tumors. When we exposed normal breast cells to estrogen and an anti-estrogen, we altered expression of this gene.

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Introduction

Breast cancer is one of the leading causes of cancer death. The purpose of this grant was to identify new Vitamin D_3 analogs in combination with other compounds that could inhibit proliferation, induce differentiation and prevent invasive growth qualities of breast cancer both *in vitro* and *in vivo* as well as to attempt to identify new genes which are modulated in breast cancer especially after association with Vitamin D analogs and to look for the ability of T lymphocytes (TALL-104) plus/minus Vitamin D analogs to inhibit the growth of breast cancer cells.

We have completed several *in vitro* and *in vivo* studies of novel vitamin D_3 analogs used alone and in combination against breast cancer. In our prior studies, we synthesized a series of novel 20-cyclopropyl-cholecalciferol vitamin D_3 analogs that were found to be potent inhibitors of clonal growth of MCF-7 breast cancer cells. We examined a new class of Vitamin D analogs that have a novel 5,6-trans motif; the most potent of these analogs affect cell cycle regulatory mechanisms, upregulate expression of cyclin dependent kinase (CDK) inhibitors, inhibit tolomerase activity, and induce expression of a novel candidate tumor suppresser gene. We examined the use of vitamin D analogs in combination with All-trans-retinoic acid in human breast tumors in BNX mice and found an additive effect which decreased tumor mass nearly 3-fold with minimal toxicity. Due to the dearth of *in vivo* studies examining the long term effects of Vitamin D analogs, we administered unique analogs for approximately one year to Balb/C mice and performed extensive toxicity analyses which revealed that these compounds were well tolerated with minimal toxicity.

We have shown that a peroxisome proliferator/activated receptor gamma (PPARγ) (Troglitazone) in combination with All-trans-retinoic acid (ATRA) a ligand for retinoic acid receptor, significantly decreased proliferation and induced differentiation and apoptosis in human breast cancer cells *in vitro* and *in vivo*. Pursuant to these studies, we initiated a clinical trial to examine the safety and efficacy of the synthetic ligand of the peroxisome proliferator/activated receptor gamma (PPARγ) Troglitazone and All-trans-retinoic acid in patients with metastatic breast cancer. This trial is still ongoing.

We have demonstrated that the organic arsenical, Melarsoprol, has significant activity in breast and prostate cancers. This compound may have efficacy as a novel treatment for breast cancer.

We continue to search for genetic alterations of breast cancer; our studies include examination of a mutation of the $p16^{INK4A}$ binding domain of the CDK4 gene and evaluation of the novel tumor suppressor gene DPC4/SMAD4 in diverse types of cancers, including human breast cancer. To better understand the role of cyclin dependent kinase inhibitors in breast cancer, and a variety of other neoplasms were examined for $p21^{WAF1}$, $p27^{KIP1}$, $p15^{INK4B}$, $p16^{INK4}$, $p18^{INK4C}$ and 19^{INK4} alterations.

We have demonstrated that $1,25OH_2$ -D_3 (vitamin D_3) modulates BRCA1 expression in a panel of breast and prostate cancer cell lines and that the extent to which either vitamin D_3 or its analogs modulates BRCA1 is often proportional to their ability to be clonally inhibited. Reducing vitamin D receptor content in one cell line reduces the clonal sensitivity and ability to induce BRCA1. These data suggest that BRCA1 protein expression is an important pathway for controlling cell proliferation in both breast and prostate cells. We speculate that some aspect of Vitamin D_3 signaling, such as co-activator is lost in these cells that selectively reduce transactivation of genes that are critical to controlling cellular proliferation.

Body

The active vitamin D_3 metabolite 1,25-dihydroxyvitamin D_3 (compound C) is an important modulator of cellular proliferation and differentiation in a variety of normal and malignant cells. Most breast cancer cell lines and more than 80% of breast tumors express high affinity intracellular vitamin D receptors (VDR) (1-4). The hypercalcemic effect of vitamin D_3 has limited its clinical utility. Vitamin D_3 analogs have been developed that inhibit cellular proliferation and induce differentiation without the attendant hypercalcemia (6-8). Vitamin D_3 analogs and Taxol are able to inhibit the *in vitro* growth of a variety of malignant cells including breast cancer cells. These compounds decrease growth by different mechanisms and have non-overlapping toxicities. We examined three vitamin D_3 analogs to inhibit MCF-7 human mammary cancers in BNX triple immunodeficient mice, either alone or in combination.

One of the exciting new analogs identified to date is 1,25-$(OH)_2$-16-ene-23-yne-26,27-F_6-19-nor-D_3 (compound LH). This potent analog reduced the development of breast cancer in nitroso-N-methylurea-treated rats (10). Furthermore, dose-response studies showed that it was one of the most potent vitamin D_3 analog in suppressing clonogenic cancer growth, being able to suppress at 10^{-11} M greater than 50 % clonal proliferation of the MCF-7 and SK-BR-3 breast cancer cells. The analog increased the proportion of cancer cells in G_0/G_1 phases and decreased those in the S phase of the cell cycle (9). Pulse-exposure studies showed that three day exposure to LH (10^{-7} M) in liquid culture was able to achieve a 50% inhibition of MCF-7 clonal growth in soft-agar in the absence of analog suggesting that inhibition of growth mediated by Compound LH is irreversible (9). Further studies have found that the cyclin dependent kinase inhibitor known as $p27^{Kip1}$ is induced at high levels by Compound LH in the MCF-7 and SK-BR-3 breast cancer cells (9).

The analog 24a,26a,27a-trihomo-22,24-diene-$1,25(OH)_2D_3$ (Compound EB1089) has a wide spectrum of anti-cancer activities *in vitro* including breast cancer cells (11-14); the analog is between 7-50-fold more potent than the parental $1,25(OH)_2D_3$ (Compound C) in vitro against cancer cells. It appears to have similar affects on serum calcium levels as compared to $1,25(OH)_2D_3$ (11). A Phase I study in patients with advance breast cancer showed that EB1089 can be given with predictable affects on cancer

metabolism (14). A Phase II trial of this novel vitamin D_3 analog is currently underway in patients with breast carcinoma.

The taxanes are an important new class of anticancer agents that exert their cytotoxic effects through a unique mechanism. Paclitaxel (Taxol) stabilizes microtubules and inhibits their depolymerization to free tubulin. It can block mitosis, induce extensive formation of microtubule bundles in cells, and cause multinucleation of cells during interphase (15-17). Taxol, the first taxane in clinical trials, is active against a broad range of cancers that are generally considered to be refractory to conventional chemotherapy. This has led to the regulatory approval of Taxol in many countries for use as palliative therapy of patients with ovarian and breast cancers resistant to chemotherapy. Taxol was discovered as part of a National Cancer Institute program in which extracts of thousands of plants were screened for anticancer activity (15). Taxol-based combinations, especially those with doxorubicin or cisplatin, appear promising for further study (18,19).

The vitamin D_3 compounds and taxol have non-overlapping toxicities. To our knowledge, no one has studied the ability of the combination of a vitamin D_3 analog and Taxol to inhibit growth of human breast cancer in vivo. We report that this combination of therapy is efficacious in inhibiting the growth of MCF-7 tumors in BNX nude mice.

In our studies animals were bilaterally, subcutaneously injected with 10^6 MCF-7 cells/tumor in 0.1 ml Matrigel (Collaborative Biomedical Products, Bedford, MA). Before injection of cells, the animals received 300 rads whole body irradiation. Mice were divided randomly into eight groups of five mice each:Group A:nontreatment (control); Group B:Taxol; Group C:Compound C; Group D: Compound C + Taxol; Group E:Compound LH; Group F:Compound LH + Taxol; Group G:EB1089; and Group H:EB1089 + Taxol.

Vitamin D_3 analogs were administered intraperitoneally every other day at the following doses: Compound C, 0.05 µg/mouse; Compound LH,0.0125 µg/mouse; and EB1089, 0.05 µg/mouse. The doses were chosen after a series of initial experiments determined the highest dose of the vitamin D_3 that could be given without causing hypercalcemia. Taxol (25 mg/kg/mouse) was administered intraperitoneally once a week. The dose was chosen from the report of Kalechman,K. et.al (19). One day after tumor injections, mice were treated with either vitamin D_3 analogs alone, Taxol alone, or the combination of a vitamin D_3 analog and Taxol. During the experiment, four mice died: one in the Compound C + Taxol group; one in the Compound LH + Taxol group; and two in the EB1089 + Taxol group. The cause of their deaths was unknown.

Tumors were measured every week with vernier calipers. Tumor size index was calculated by the formula: a x b x c, where a is the length and b is the width and c is the height in millimeters. Serum calcium values were measured on days 20 and 68 by

atomic absorption spectrophotometry (Perkin-Elmer 560) and a modification of the calcium o-cresolphthalein complexone complexometric reaction (Dupont Analyst Benchtop Chemistry System, Dade International).

At the end of the experiment, animals were killed by CO_2 asphyxiation and tumor weights were measured after careful resection, and blood was also collected from the orbital sinus for chemistry and blood analysis. Chemistries and blood analyses were measured by Dupont Analyst Benchtop Chemistry System, Dade International, Newark, DE and by Serono-Baker 9000 Diff, Biochem Immuno-Systems, Allentown, PA, respectively.

At completion of the trial, all the treatment groups had statistically significantly smaller tumors than the non-treated group. Administration of vitamin D_3 analogs alone remarkably suppressed the growth of the tumors. The most potent single agent was EB1089 (Group G). The second in potency was Compound LH (Group E), followed by Compound C. The antitumor effect of vitamin D_3 analogs appeared greater than that of Taxol and the enhanced activity was observed when vitamin D_3 analog and Taxol were administerd together. In each case, the combination of a vitamin D_3 compound and Taxol suppressed tumor growth greater than either alone.
The results were similar when the effect of vitamin D_3 analogs and Taxol were evaluated by tumor weight at the conclusion of the study (Figure 1). Again, EB1089 was the most potent single agent, and the combination of a vitamin D_3 compound and Taxol was more potent than either alone. Tumor weights in the combined treatment groups were approximately 30% to 50% of those in the no treatment group. All the treatment groups were statistically different from Group A (control, $p<0.01$). In addition, Group H (EB1089 + Taxol) was statistically different from Groups D (Compound C + Taxol) and F (Compound LH + Taxol).

The dose of these vitamin D_3 analogs that caused a remarkable inhibition of the size and weight of breast cancer did not elevate the level of the serum calcium (normal 8.5-10.5 mg/dl). We believe that initial calcium values were lower in all mice including controls than later in the study as a consequence of using two different methods of measurement. In the first measurement, we used atomic absorption spectrometry and in the second we used a modification of the calcium o-cresolphthalein complexone complexometric reaction.

During the study, all mice were weighed once per week (Figure 2). Each of the cohorts gained in weight, but groups D (Compound C + Taxol) and G (EB1089) had statistically lower body weights than the non-treated group. The body weights in these three groups were 82-87% of that in the control Group A. The body weights of the other cohorts were not statistically different compared to the untreated control Group A. In general, each of the mice looked healthy.

The present data show that the vitamin D_3 analogs and Taxol had potent anti-breast cancer activity *in vivo* without causing hypercalcemia and other major side-effects. Combined treatment of the MCF-7 human breast cancer cells resulted in a stronger inhibition than treatment with either a vitamin D_3 compound or Taxol alone.

Antiestrogen therapy is the pivotal endocrine therapy of breast cancer (20). However, breast cancer patients whose tumors do not express estrogen receptors constitute 30-40% of breast cancer patients (5); and they have a significantly worse prognosis than those with estrogen receptors (21). Furthermore, resistance to antiestrogen therapy frequently occurs (22); in these situations, treatment with vitamin D_3 might be useful.

The $1,25(OH)_2D_3$ and its analogs can inhibit tumor growth by a variety of mechanisms, including regulation of angiogenesis, apoptosis, tumor invasiveness and G_0/G_1 cell cycle arrest as a result in part of the enhanced expression of the cyclin dependent kinase inhibitors known as $p21^{WAF1}$ and $p27^{Kip1}$ (23-27). Despite promising antitumor activity of $1,25(OH)_2D_3$ *in vitro*, its calcemic toxicity *in vivo* limits the doses that can be given. The vitamin D_3 analogs LH and EB1089 have almost the same growth inhibitory action as Compound C, but 50-100-fold lower concentrations of these analogs were required or this anticancer activity. In contrast, *in vivo* studies have shown that the calcemic activity of the EB1089 analog was lower than $1,25(OH)_2D_3$ (11,28-30). The Compound LH had slightly higher calcemic activity than $1,25(OH)_2D_3$ (11). Our data show that the growth inhibitory action of the three vitamin D_3 compounds was statistically greater than that of the non-treatment group. Moreover, each of these cohorts had inhibition of tumor growth without hypercalcemia.

Taxol is one of the most important new cytotoxic agents to be introduced for the management of breast cancer in several years (15-19). Combinations of Taxol with various cytotoxic agents are being actively explored (31-34). As expected,the present data show that taxol has an anti-breast cancer effect *in vivo*. The combination of one of the vitamin D_3 compounds with Taxol remarkably suppressed the growth of human breast cancer cells *in vivo* (Figure 1). This was shown most impressively when examining the tumor weights at the conclusion of the study, which decreased 70% in the mice that received the combination of EB1089 with Taxol as compared to that in the diluant-treated control group.

Chemotherapy of many stages of breast cancer is still based on the combined use of three major classes of anticancer drugs: alkylating agents, antimetabolites, and anthracycline antibiotics. Nevertheless, these combined chemotherapies are associated with overlapping toxicities and are not completely effective. Therefore, combinations of different forms of therapy including biologic modifiers such as vitamin D_3 analogs combined with Taxol, as well as antiestrogens, and retinoids may be worthwhile.

Taxol exerts its cytotoxic effects through a unique mechanism of microtubules

stabilization resulting in blockade of mitosis (16,17,35). The vitamin D_3 compounds are lipid soluble and freely enter the cell. They bind and activate the vitamin D_3 receptors, allowing efficient interaction with vitamin D response elements thus modulating the expression of various genes. Despite intense research, the exact mode of action by which vitamin $1,25(OH)_2D_3$ and its analogs inhibit cancer cells growth remains largely unknown (36). They can lead to cell cycle arrest with elevation in levels of $p21^{WAF1}$ and $p27^{KIP1}$ cyclin dependent inhibitors. Taken together, the vitamin D_3 compounds and taxol probably inhibit proliferation of cancer cells including those of the breast by different mechanisms. Furthermore, the toxicities of the two therapies are clearly different, with the vitamin D_3 compounds potentially producing hypercalcemia and the taxols having the ability to cause hematopoietic cytopenias.

The prevention of cancer (chemoprevention) is clearly more cost effective than the treatment of an established cancer and may represent the best approach to this disease. We (and others) have shown that activation of the peroxisome proliferator activated receptor gamma (PPARγ) by thiazolidinediones, including the synthetic ligand Troglitazone (TGZ), inhibits cultured breast cancer cell lines (37,38). All-trans-retinoic acid (ATRA) also inhibits *in vitro* proliferation of breast cancer cells and the combination of TGZ and ATRA caused significant apoptosis of MCF-7 breast tumors in mouse models without significant toxicity (38).

Retinoids (RA) mediate their activity via the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). PPARγ is a memeber of the nuclear hormone receptor superfamily that includes retinoic acid receptors (RAR and RXR) and thyroid hormone receptors. PPARγ heterodimerizes with RXR and binds to DNA, resulting in expression of genes associated with many aspects of differentiation, cellular development and general physiology (39,40). The PPARγ ligand TGZ is useful for the treatment of Type 2 diabetes and has been used to treat over 1 million individuals with this disease. These agents may enhance differentiation of adiposytes and thus may be associated with upregulation of their glucose pumps, however, the exact mechanism of action is unclear. All-trans-retinoic acid, an RAR specific ligand, selectively inhibits growth of ER-positive breast cancer cells (41,42) and is also effective in preventing mammary carcinogenesis in rodents (43). The lack of toxicity for most individuals receiving TGZ for adult onset diabetes, as well as the lack of adverse effects of several RXR ligands including 9-cis retinoic acid, makes the combination of TGZ and a RXR analog an attractive combination for *in vivo* chemoprevention trials. Furthermore, there are several new thiazolidinediones now available; these compounds do not appear to have the idiosyncratic liver toxicity that occurs rarely with administration of TGZ.

The murine mammary gland organ culture system effectively evaluates the effects of potential chemopreventive agents to inhibit the induction of preneoplastic lesions (44-47). Mammary glands of BALB-C mice are placed in organ cultures containing a variety

of growth proliferative factors and hormones and are treated with the carcinogen 7,12-dimethylbenzine[a]anthracene (DMBA) to induce preneoplastic lesions. The mammary epithelial cells isolated from these lesions, are placed into synergistic hosts and subsequently develop adenocarcinomas. Using this technique over 150 different chemopreventive agents have been tested. This assay is highly reproducible and provides a good correlation with the efficacy of a chemopreventive agent in both *in vitro* and *in vivo* models. We have used this model system to analyze the efficiency of TGZ and/or a retinoid to prevent the formation of DMBA-induced mammary lesions (MAL) in a murine mammary gland organ culture model.

In our studies we found that combining a PPARγ with a ligand specific for RXR (LG10069) enhanced the suppression of development of mammary lesions. Previous studies have shown that simultaneous activation of both receptors can result in synergistic activity in several assays of cultured cells as well as augmented *in vivo* anti-diabetic activity (48). Furthermore, we have previously shown that a PPARγ ligand and a RXR ligand can have enhanced antiproliferative effects against both breast and prostate cancer cells (38,49). Previous studies have shown that a RXR-specific agonist (LG10069) had chemopreventive activity against chemically induced rat mammary tumors (50). However, such activity for LG10068 has not been reported.

We examined the effect of TGZ combined with a RXR ligand (LG10068). The RXR ligand (10^{-7}-10^{-8} M) was unable to inhibit DMBA-induced mammary lesions shows a DMBA-induced mammary lesion, and TGZ (10^{-6}) in this series of experiments inhibited mammary lesions only by approximately 14%. However, when the two were combined, the percent inhibition of development of breasts with abnormalities was 85%, showing that the two ligands together were clearly more effective that either alone. The PPARγ heterodimerizes with RXR, and each can simultaneously bind to their ligand resulting in enhanced activity of this activated receptor complex. These results are shown in a representative photograph of the gland. Additional studies showed that TGZ probably inhibited both the initiation as well as the progression of the DMBA-induced lesions. Further studies are required to determine the target genes associated with this anti-cancer activity.

This is the first report showing the possibility of a PPARγ ligand having chemopreventive activity. Troglitazone is a relatively non-toxic compound at a wide range of concentrations, but it is a potent inhibitor of the development of preneoplastic lesions of the mammary gland in organ culture. Also, a RXR- or RAR-selective retinoid appears to enhance this chemopreventive activity; thus, the combination of a thiazolidinedione with a retinoid, such as either ATRA or LG10068, may be a good candidate for an *in vivo* breast cancer chemoprevention study. Individuals at high risk for developing breast cancer can be identified due to the recent advances in genetics and the epidemiology of breast cancer. It is these individuals who may receive the most benefit from a chemoprevention regimen containing a PPARγ ligand combined

with a retinoid.

Molecular Alterations in Breast Cancer:

To continue our work to identify genes that are involved in the tumorigenesis of breast cancer, PCR-selected cDNA subtraction was utilized to construct a breast cancer subtracted library. Differential screening of the library isolated the growth factor inducible immediate-early gene Cyr61, a secreted, cysteine-rich, heparin binding protein that promotes endothelial cell adhesion, migration and neovascularization. Northern analysis revealed that Cyr61 expression correlated with invasiveness and tumorgenicity in breast tumor cells. Cyr61 is expressed highly in invasive, ER negative breast cancer cell lines MDA-MB-231, SK-BR-3 and MDA-MB-157, at very low level in less tumorgenic, ER positive cells MCF7, T47D and BT-20, and barely detectable in normal breast cell MCF12A. Significantly, high expression of Cyr61 was found in about 40% breast tumor biopsies tested. Interestingly, expression of Cyr61 in breast cells is modulated by both estrogen and antiestrogen in a time- and dose-dependent manner. Expression of Cyr61 increased 8-12 fold in MCF12A and 3-5 fold in MCF7 after 48 hr estrogen treatment. The induction of Cyr61 was blocked by tamoxifen, an estrogen receptor inhibitor. These results suggest that Cyr61 may play a role in the progression of breast cancer and may be involved in estrogen-mediated tumor development.

Our data demonstrate that the combination of a vitamin D_3 analog and Taxol markedly inhibited the growth of human breast cancer cells (MCF-7) *in vivo* without causing either hypercalcemia, hematopoietic cytopenias or other major side-effects. This combination has the potential for treatment of breast cancer patients, especially in the adjuvant setting.

Chemoprevention of breast cancer is an active area of basic science and clinical investigation. Our work is the first report showing the possibility of a PPARγ ligand having chemopreventive properties. Furthermore, an RXR-selective ligand appears to enhance this activity. Because both of these drugs possess relatively minor toxicity profiles, this combination may be a good candidate for an *in vivo* breast cancer chemoprevention study.

Cyr61 has previously been identified in other tissue types and has been associated with angiogenesis and metastasis. We have found that selected breast cancer cell lines as well as an array of fresh breast cancers have prominent expression of this gene. Interestingly, normal breast tissue did not express this gene at all. However, the gene was activated when the normal breast cells were exposed to estrogen, and expression was blocked when exposed to an estrogen receptor inhibitor (tamoxifen). Our studies of Cyr61 may have important implications for our understanding of the progression of breast cancer.

Key Research Accomplishments:

- Identified new Vitamin D analogs that can inhibit proliferation and induce apoptosis in breast cancer cells both in vitro and in vivo.
- Identified breast cancer genes that appear to be important in breast cancer including Cyr61 which is modulated by Vitamin D as well as by estrogen and other nuclear hormone receptor ligands.
- Identified additional genes whose expression is not altered in breast cancer including thymosin B4.

Reportable outcomes:

1. Lee NE. Williard PG. Brown AJ. Campbell MJ. Koeffler HP. Peleg S. Rao DS. Reddy GS. Synthesis and biological activities of the two C(23) epimers of 1 alpha, 23,25-trihydroxy-24-oxo-19-nor-vitamin D(3): novel analog of 1 alpha,23(s),25-trihydroxy-24-oxy-vitamin D(3), a natural metabolite of 1alpha,25-dihydroxyvitamin D(3). Steroids. In press.

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Conclusions:

In summary, I think we have shown fairly clearly that Vitamin D analogs as well as other nuclear receptor ligands have a clear antiproliferative effect on breast cancer cells. Furthermore, they can induce apoptosis and some differentiation. In addition, these compounds appear to synergize with some chemotherapy, such as Taxol, to decrease the growth of human breast cancer as shown by xenographs growing in mice. Although, it is unlikely that the Vitamin D analogs would be used as primary therapy, I could well envision that they would be tried in the adjuvant setting because of their antiproliferative effects as well as their low toxicities. At this time, clinical studies are ongoing with one of the analogs known as EB1089 and an other analog is undergoing phase I testing (125dihydroxy16ene-23yne-Vitamin D_3).

STATEMENT OF WORK

Specific Aim 1: We have performed extensive studies of novel vitamin D_3 analogs as single agents or in combination for their ability to inhibit clonal proliferation, and induce apoptosis and differentiation of breast cancer cells.

Specific Aim 2: We have studied the effects of vitamin D_3 analogs in our *in vivo* models.

Specific Aim 3: We have identified new genes associated with the tumorigenesis of breast cancer.

Specific Aim 4: We continue our collaboration with Dr. Daniela Sanitoli to study the

effects of TALL-104 cells *in vitro* and *in vivo* in breast cancer cells.

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19-nor-Hexafluoride Analogue of Vitamin D_3: A Novel Class of Potent Inhibitors of Proliferation of Human Breast Cell Lines[1]

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ABSTRACT

Breast cancer cells express vitamin D_3 receptors and 1,25-dihydroxyvitamin D_3 suppressed growth of these cells. We have synthesized six novel vitamin D_3 analogues to identify those with expanded capacity to inhibit the proliferative ability of breast cancer cells. These analogues incorporated many of the structural motifs shown previously to have antiproliferative activity in several cell types. Six breast cancer cell lines were used as targets. Dose-response studies showed that each of the analogues had antiproliferative activities, and LH [1,25-$(OH)_2$-16-ene-23-yne-26,27-F_6-19-nor D_3] was the most potent analogue, suppressing at 10^{-11} M greater than 50% clonal proliferation (ED_{50}) of the MCF-7 and SK-BR-3 breast cancer cells, increasing the proportion of MCF-7 cells in the G_0-G_1 phase, and decreasing those in the S phase of the cell cycle. Pulse-exposure studies showed that a 3-day exposure to LH (10^{-7} M) in liquid culture was adequate to achieve a 50% inhibition of MCF-7 clonal growth in soft agar in the absence of the analogue, suggesting that the growth inhibition mediated by LH is irreversible. The cyclin-dependent kinase inhibitor known as *$p27^{Kip1}$* helps regulate the cell cycle and can mediate growth arrest in response to extracellular growth inhibitors. The analogue LH (10^{-7} M) induced elevated expression of *$p27^{Kip1}$* in MCF-7 and SK-BR-3 cells. Taken together, these results indicate that LH is an extremely potent vitamin D_3 analogue markedly inhibiting clonal growth of MCF-7 and SK-BR-3 cells with concomitant cell cycle arrest at G_0-G_1 and increased expression of *$p27^{Kip1}$*. Compound LH is worthy of *in vivo* analysis for possible future clinical trials.

INTRODUCTION

Breast cancer is the most common malignant disease of women in the United States. Improvement in hormonal and cytotoxic therapies have not led to either a major lengthening of remissions or increase in cures in advanced breast cancer. Antiestrogens have provided the most effective endocrine therapy for advanced breast cancer (1). The 1,25 D_3[3] is a member of the seco-steroid family. Most breast cancer cell lines and more than 80% of breast tumors expressed high affinity VDRs (2–4). Reminiscent of estrogen receptor data, patients with primary carcinoma of the breast who were VDR positive had significantly longer disease-free survival than those with VDR-negative tumors (5). The 1,25 D_3 and its analogues inhibit proliferation of breast cancer cells *in vitro* (6–8). Likewise, 1,25 D_3 and its related analogues decreased the progression of breast cancer and other carcinomas *in vivo* (9–11), inhibited metastatic spread of tumors cells (2, 12–14), and promoted differentiation of breast cancer cells as well as other varieties of cancer (5, 8, 15–18). However, the calcemic side effects of 1,25 D_3 have prevented its application as a pharmaceutical agent. Synthesis of analogues of 1,25 D_3 with potent antiproliferative and differentiation activity against cancer cells with decreased risk of inducing hypercalcemia has been reported (9, 11, 12, 19–21).

Previously, we have studied the *in vitro* biological activities and mechanism of action of four potent 1,25 D_3 analogues (KH1060, 20-epi-1,25$(OH)_2$-D_3, 1,25$(OH)_2$-16-ene-D_3, and V) on the proliferation and differentiation of six breast cancer cell lines. In that study, KH1060 was the most potent 1,25 D_3 analogue inhibiting clonal growth of four breast cancer lines, with decreased bcl-2 and cell cycle arrest at G_0-G_1 (22).

$p27^{Kip1}$ is a recently cloned cyclin-dependent kinase inhibitor associated with arrest of the cell cycle (23, 24). We have examined for *$p27^{Kip1}$* mutations in 36 primary breast cancers and 9 breast cancer cell lines (25). Only two point mutations were found in the primary tumors. Additional studies showed that overexpressed Kip proteins caused cell cycle arrest, and expression of *$p27^{Kip1}$* was up-regulated by exposure of the cells to several antimitogens (23, 26–30). In this study, we have shown that LH was the most potent of a new series of 1,25 D_3 analogues in mediating the inhibition of clonal growth of MCF-7 and SK-BR-3 breast cancer cells associated with cell cycle arrest at G_0-G_1 and increased expression of *$p27^{Kip1}$*.

MATERIALS AND METHODS

Cell Lines. The breast cancer cell lines (MDA-MB-436, MCF-7, SK-BR-3, BT-474, BT-20, and MDA-MB-231) were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM or McCoy's media (Life Technologies, Inc., Grand Island, NY) containing 10% bovine fetal serum, according to the recommendations of American Type Culture Collection, in culture flasks with vented filter caps (Costar, Cambridge, MA).

Vitamin D_3 Compounds. The vitamin D_3 compounds were dissolved in absolute ethanol at 10^{-3} M as stock solution, which were stored at −20°C and protected from light. The analogues C, Y, LH, KS, KY, KW, and LA were synthesized by Hoffmann LaRoche, Inc. (Fig. 1).

Clonogenic Assay in Soft Agar. Breast cancer cells were cultured in a two-layer soft agar system for 14 days, as described previously (31).

Pulse-Exposure Experiments. The MCF-7 cells were exposed to analogue LH (10^{-7} M) for various durations. After incubation, the cells were carefully washed twice, counted, and plated into 24-well plates for soft agar colony assay.

Cell Cycle Analysis by Flow Cytometry. Cell cycle analysis was performed on breast cancer cells incubated for 72 h with or without LH at 10^{-7} M. The methanol-fixed cells were incubated for 30 min at 4°C in the dark with a solution of 50 μg/ml propidium iodide, 1 mg/ml RNase (100 units/ml; Sigma Chemical Co.), and 0.1% NP40 (Sigma). Analysis was performed immediately after staining using the CELLFit program (Becton Dickinson), whereby the S phase was calculated with a Rfit model.

Western Blot Analysis. Cultured cells were washed twice with PBS and then lysed in 1 ml/10^7 cells of 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin for 30 min at 0°C. Insoluble material was removed by centrifugation at 14,000 rpm at 4°C

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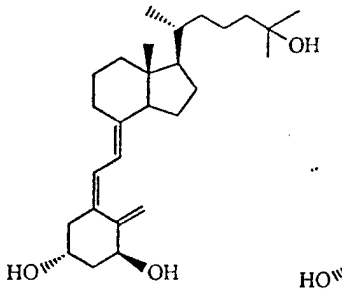
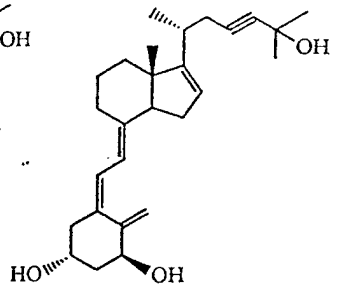
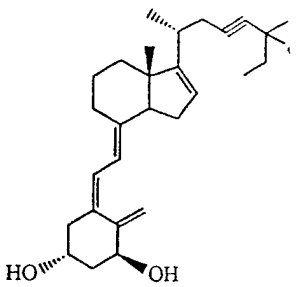
[3] The abbreviations used are: 1,25 D_3, 1,25-dihydroxyvitamin D_3; VDR, vitamin D_3 receptor; C, 1,25$(OH)_2$-D_3; LA, 1,25-$(OH)_2$-16,22R,23-triene D_3; LH, 1,25-$(OH)_2$-16-ene-23-yne-26,27-F_6-19-nor D_3; V, 1,25-$(OH)_2$-16-ene-23-yne D_3; KS, 1,25-$(OH)_2$-16-ene-23-yne-26,27-bishomo D_3; KW, 1,25-$(OH)_2$-22E, 24E-diene-24-homo,26,27-F_6-D_3; KY, 1,25-$(OH)_2$-16,23E-diene-26,27-F_6-20-epi-D_3; KH1060, 20-epi-22-oxa-24a,26a,27a-tri-homo-1,25$(OH)_2$-D_3; CDK/cdk, cyclin-dependent kinase.



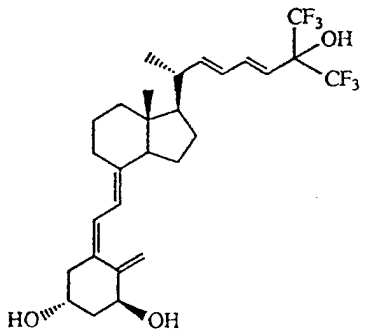
C. 1,25-$(OH)_2D_3$



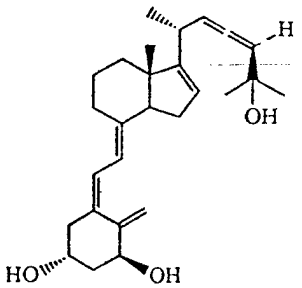
V. 1.25-$(OH)_2$-16-ene-23-yneD_3



KS. 1.25-$(OH)_2$-16-ene-23-yne-26,27-bishomoD_3

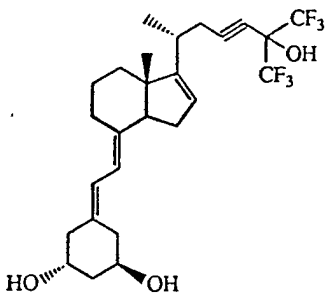


KW. 1,25-$(OH)_2$-22E,24E-diene-24-homo-26,27-F_6D_3

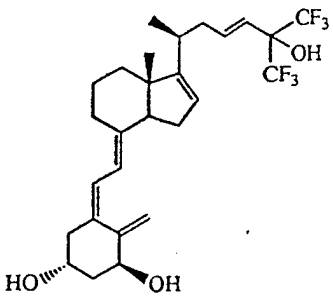


LA. 1,25-$(OH)_2$-16,22R,23-triene D_3

Fig. 1. Structures and code names of the novel vitamin D_3 analogues examined in this study.



LH. 1,25-$(OH)_2$-16-ene-23-yne-26,27-F_6-19-nor D_3



KY. 1,25-$(OH)_2$-16,23E-diene-26,27-F_6-20-epi D_3

for 10 min. Protein concentrations were determined using a Bio-Rad kit. Proteins (40 μg) were size fractionated under denaturing conditions on 12.5% SDS-running gel and transferred to Millipore membrane and exposed without drying to the X-ray film overnight. The $p27^{Kip1}$-specific band was detected by Western blot hybridization of the membrane with purified anti-$p27^{Kip1}$ antibody and detection of the signal was with the ECL system (Amersham). A rabbit polyclonal anti-serum specific for human $p27^{Kip1}$ (Santa Cruz) was used for Western blot analysis.

RESULTS

Effect of Vitamin D_3 Analogues on Clonal Proliferation of Breast Cancer Cell Lines. Breast cancer cells were cloned in soft agar in the presence of vitamin D_3 analogues at 10^{-11} to 10^{-7} M. Dose-response curves were drawn (Fig. 2), and the effective dose that inhibited 50% colony formation (ED_{50}) was determined (Table 1). The 1,25 D_3 analogues were effective in inhibition of clonal proliferation of two of the six breast cancer cell lines (MCF-7 and SK-BR-3; Fig. 2, *a* and *b*).

The BT-474 and MDA-MB-231 breast cancer cells were fa resistant to the vitamin D_3 analogues, and the MDA-MB-436 BT-20 were even more resistant (Fig. 2, *c–f*). For the two sensi breast cancer cell lines (Fig. 2, *a* and *b*) as well as MDA-MB-231, LH analogue was the most potent compound. The LH analo achieved an ED_{50} of 8×10^{-11} and 8×10^{-10} M for SK-BR-3 MCF-7, respectively (Table 1).

Pulse-Exposure Experiments. The MCF-7 cells were exposec the analogue LH (10^{-7} M) for various durations, washed three ti to remove the analogue, and plated in soft agar; and colony num was enumerated on day 14 (Fig. 3). Fifty % of the clonogenic c were inhibited by 3 days of exposure to analogue LH, suggesting this compound was capable of mediating an irreversible inhibitio the growth of these cells.

Cell Cycle Analysis. Effect of LH on the cell cycle of br cancer cells was determined by studying the MCF-7 cells. These c had a significant increase in the number of cells in the G_0-G_1 phas the cell cycle [70 ± 2% with LH (10^{-7} M) for 72 h, 52 ± 1.7%

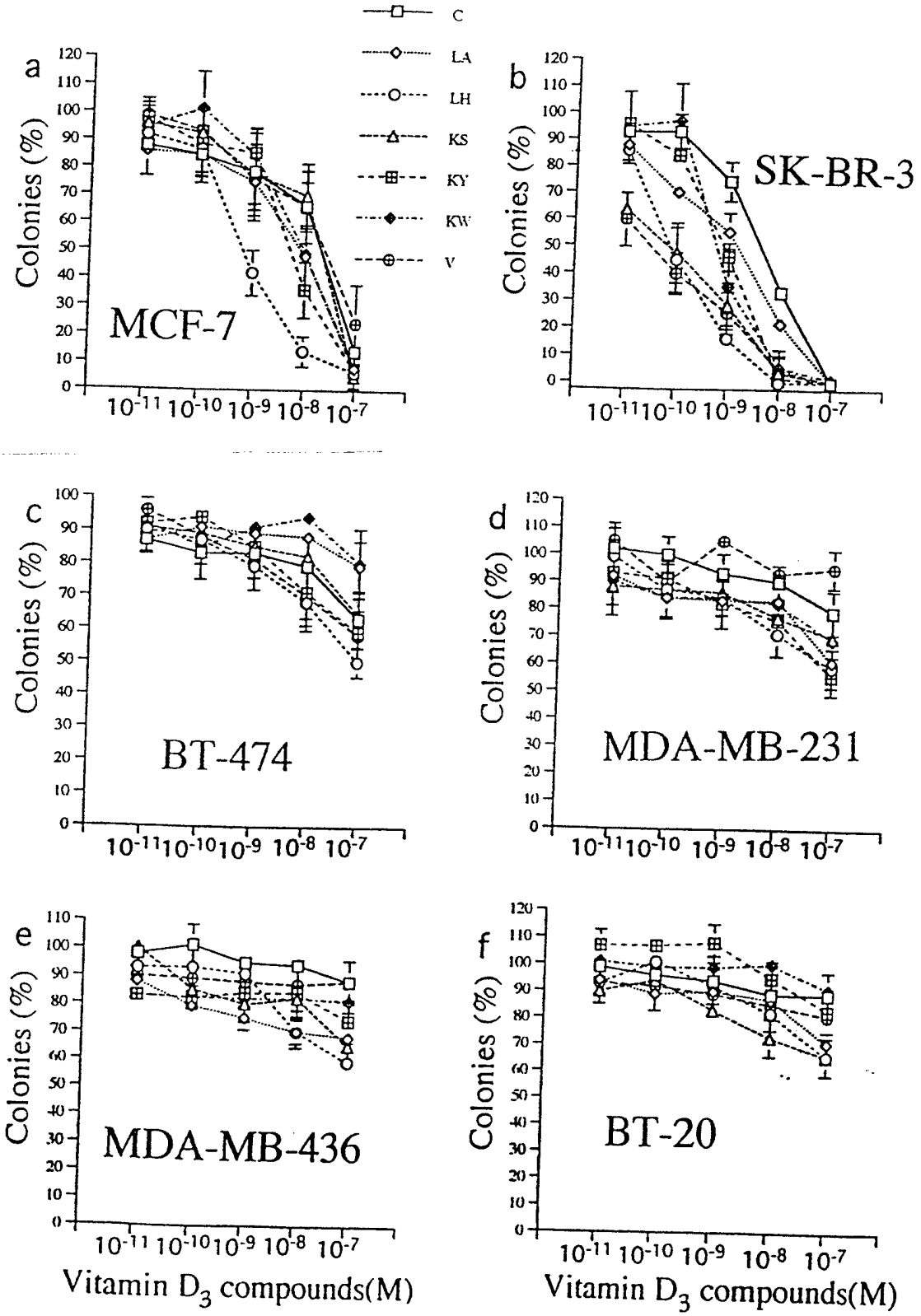


Fig. 2. Dose-response effects of vitamin D_3 compounds on clonal proliferation of breast cancer cell lines. Results are expressed as a mean percentage of control plates containing no vitamin D_3 compounds (means of at least three experiments with triplicate dishes; *bars*, SD).

control cells] with a concomitant decrease in the S phase [16 ± 2% with LH (10^{-7} M) for 72 h, 35 ± 2.4% in control cells] ($P < 0.05$; Fig. 4).

Increased Levels of $p27^{Kip1}$ Induced in MCF-7 and SK-BR-3 Cell Lines during Exposure to Analogue LH. The MCF-7 and SK-BR-3 cells had a moderate level of expression of $p27^{Kip1}$ (Fig. 5, *Lanes 2* and *4*) as determined by Western blot analysis. Exposure of these cells to LH (10^{-7} M) resulted in a modest increase in expression of $p27^{kip1}$ with levels increasing 40% in SK-BR-3 cell line at day 3 of exposure and 30% in MCF-7 cells at day 6 of exposure to the analogue (Fig. 5). Similar findings were found on repeat experiments.

Table 1 *Inhibition of clonal proliferation of breast cancer cell lines by vitamin D_3 analogues*

Breast cancer cell lines	Inhibition of clonal proliferation, ED_{50} (M)[a]						
	1,25 D_3	LA	LH	KS	KY	KW	V
MCF-7	3×10^{-8}	1×10^{-8}	8×10^{-10}	3×10^{-8}	8×10^{-9}	1×10^{-8}	5×10^{-8}
SK-BR-3	8×10^{-9}	3×10^{-9}	8×10^{-11}	8×10^{-11}	8×10^{-10}	6×10^{-11}	8×10^{-10}
BT-474	N.R.[b]	N.R.	8×10^{-8}	N.R.	N.R.	N.R.	N.R.
MDA-MB-231	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.
BT-20	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.
MDA-MB-436	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.

[a] Dose-response curves (Fig. 2) were used to calculate the concentration of the analogues achieving a 50% inhibition (ED_{50}) of clonal growth.
[b] N.R., the ED_{50} was not reached at $\leq 10^{-7}$ M of the 1,25 D_3 analogue.

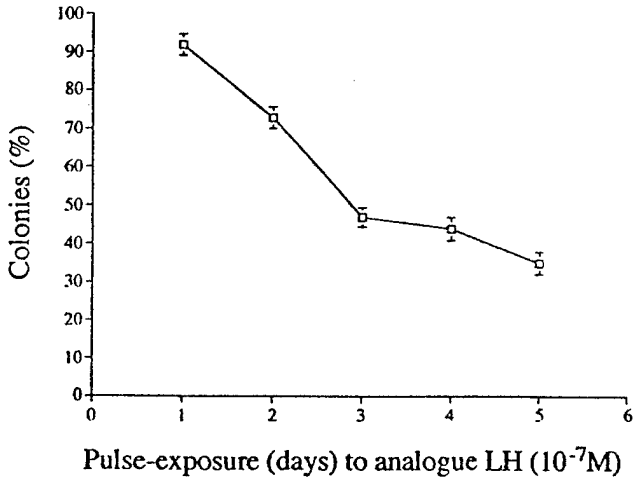


Fig. 3. Pulse-exposure of MCF-7 breast cancer cells to analogue LH. MCF-7 cells were exposed for various durations to analogue LH (10^{-7} M). The cells were then thoroughly washed three times and plated into soft agar, and colonies were counted 14 days after plating. Results are expressed as a mean percentage of control plates that received the same treatment but were not exposed to analogue (mean of three independent experiments; *bars*, SD).

DISCUSSION

The six breast cancer cell lines used in our study varied in their sensitivities to the clonal inhibitory effects of the various 1,25 D_3 compounds. The most sensitive lines, MCF-7 and SK-BR-3, were inhibited by each of the analogues. In contrast, MDA-MB-436, BT-20, BT-474, and MDA-MB-231 cells were entirely or partially resistant to the 1,25 D_3 analogues used in this study, suggesting that preclinical testing of the breast cancer cells *in vitro* may be helpful in the selection of patients for clinical trials with these analogues.

The LH was the most active analogue in this study, which has double bonds at C16, triple bonds at C23, six fluorine substitutions at C26 and C27, and removal of a methylene at C19. Previously, we have shown that hexafluoro analogues of 1,25-$(OH)_2D_3$ were 5- to 350-fold more potent than 1,25 D_3 as antileukemic agents (21). LH was also very active against prostate cancer cell lines (LNCaP, PC-3, and DU-145 cell lines; Ref. 32).

Earlier, we have shown that a 20-epi-vitamin D_3 (KH-1060) was a very potent analogue against breast cancer cell lines (22). We synthesized a 20-epi-analogue (compound KY) that combined structural motifs shown previously to be important for antiproliferative activity against cancer cells (C16 and C23 double bonds, C26 and C27 hexa-fluorines). But, the addition of these structural elements to the 20-epi backbone did not increase antiproliferative activity (Fig. 2) as compared to the 20-epi form alone (22).

One of the interesting biologically and potentially clinically relevant observations that was made in this and another study done by us recently (33) is that cancer cells of different tissues can display different sensitivities to the same vitamin D_3 analogues. For example, analogue LA is one of the most potent analogues in its inhibitio clonal growth of HL60 leukemia cells, with an ED_{50} of 2×10^{-1} whereas analogue LH had an ED_{50} of 2×10^{-10} M in the same as In contrast, analogue LA was 1 to 2 logs less effective than analo LH in its antiproliferative activity against breast and prostate ca cell lines (Table 1; Refs. 32 and 33). These results suggest that mechanism of growth inhibition of cancer cells by vitamin D_3 a logues may vary between different types of tissues.

Pulse-exposure of MCF-7 cells for 3 days to analogue LH (10^{-7} washing, plating in soft agar, and enumerating colony formation days after plating resulted in 50% inhibition of colony formati These results suggest that LH inhibited growth of breast cancer c by a mechanism other than one that is merely cytostatic. Furtherm LH increased the number of MCF-7 cells in G_1 and decreased number in S phase (Fig. 4).

Recently, several CDK inhibitor (*CDKI*) genes have been clo and they have been classified into two groups (34). One g includes the INK4 proteins: $p16^{INK4A}$ (35, 36); $p15^{INK4B}$ (36, 37– $p18^{INK4C}$; and $p19^{INK4D}$ (39). These specifically inhibit the C complexes involving CDK4 and CDK6, which are CDKs expre exclusively at the mid-G_1 phase of the cell cycle (34, 37, 39). other group consists of $p21^{Waf1}$ (40–45), $p27^{Kip1}$ (23, 24), $p57^{Kip2}$ (46, 47). These proteins are structurally and functior unrelated to the *INK4* genes. The $p27^{Kip1}$ protein has 42% amino homology with $p21^{Waf1}$ and 47% similarity with the $p57^{Kip2}$ prc within the NH_2-terminal domain, which mediates the inhibitio CDK (23, 24, 46–49). In comparison to the INK4 proteins,

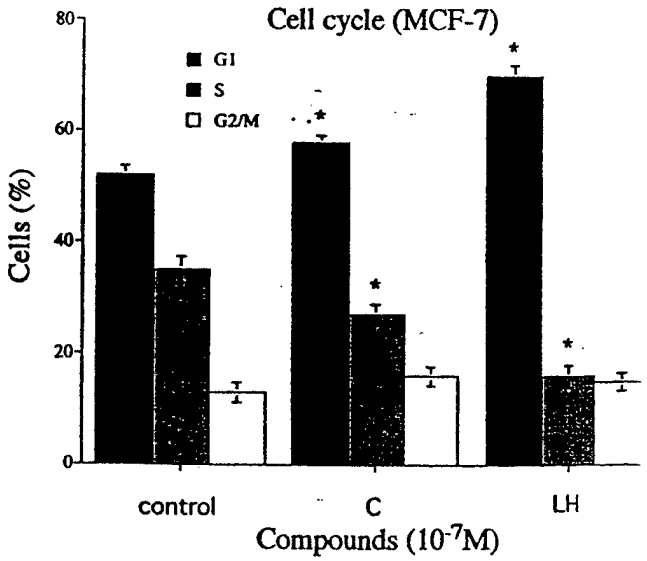


Fig. 4. Cell cycle analysis of breast cancer cells that had been cultured with e compound C [1,25$(OH)_2D_3$] or analogue LH at 10^{-7} M for 3 days. Control cells repre those not exposed to vitamin D_3 compounds. Each point represents a mean of at least t independent experiments; *bars*, SD. *, $P \leq 0.05$ as determined by Student's *t* difference from the control group.

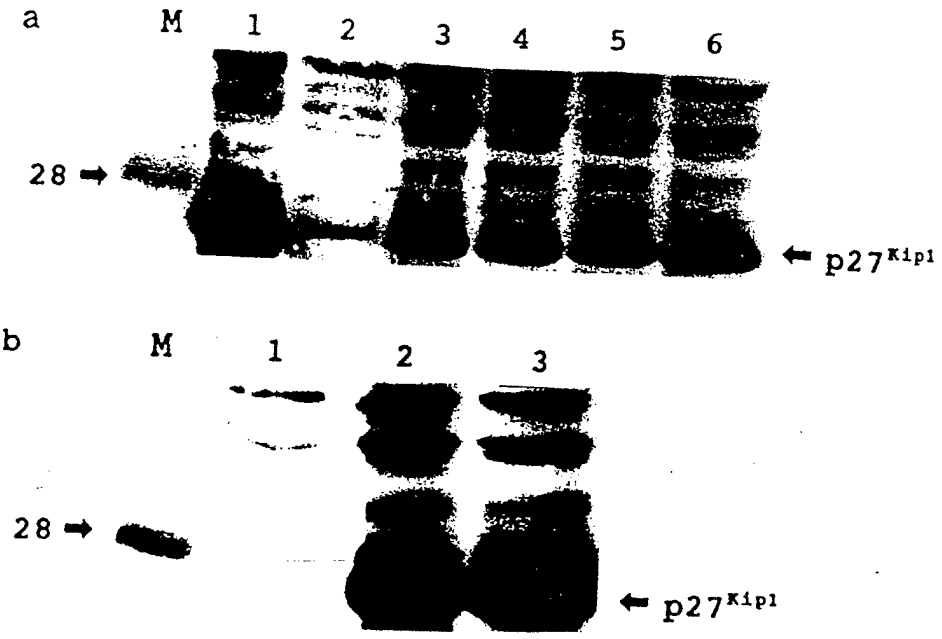


Fig. 5. Western blot analysis of $p27^{Kip1}$ levels in MCF-7 and SK-BR-3 cell lines. *a: Lane M,* marker (in thousands); *Lane 1,* MCF-7 cells exposed to LH (10^{-7} M,6 days); *Lane 2,* untreated HL-60 cells. *Lane 3,* untreated SK-BR-3 cells. *Lanes 4, 5,* and *6,* SK-BR-3 cells exposed to LH (10^{-7} M) for 1, 2, and 3 days, respectively. *b:* Lane M, marker (in thousands); *Lane 1,* untreated HL-60 cells; *Lane 2,* untreated MCF-7; *Lane 3,* MCF-7 cells exposed to LH (10^{-7} M, 6 days). Whole-cell extracts were prepared, and equal amounts of protein were loaded into each lane. Blots were then probed using the monoclonal antibody $p27^{Kip1}$ and visualized using the ECL system.

p21/p27/p57 family shows a wide specificity and spectrum of inhibitory activities. All of these proteins are able to inhibit kinase activities of preactivated G_1 cyclin E-cdk2 and cyclin D-cdk4/6, S-phase cyclin A-cdk2, as well as the mitotic cyclin B-cdc2 (26, 41, 46, 47). Overexpressed Kip proteins cause cell cycle arrest (23, 24, 48, 49). In a prior study, 1,25 D_3 (10^{-7} M, 48 h) caused a G_1 to S-phase block in parallel with an increased abundance of $p27^{Kip1}$ in HL-60 cells (30). In our study, the levels of $p27^{Kip1}$ increased at day 3 of exposure of SK-BR-3 and day 6 of exposure of MCF-7 cells to analogue LH, supporting the hypothesis that the $p27^{Kip}$ protein may be one of the mediators of the antiproliferative activity of the vitamin D_3 compounds by blocking entry of breast cancer cells into the S-phase.

In summary, we have identified a group of 1,25 D_3 analogues, including LH, with potent antiproliferative effects on the MCF-7 and SK-BR-3 breast cancer cells *in vitro*. These interesting compounds will be studied for their ability to control the growth of breast cancers *in vivo*.

ACKNOWLEDGMENTS

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EDITORIALS

Toward Therapeutic Intervention of Cancer by Vitamin D Compounds

*Moray J. Campbell, H. Phillip Koeffler**

One area of cancer chemoprevention that has been intensively studied in recent years is biologic modifiers of cancer cells that are designed to retard proliferation (*1-3*), to induce differentiation of these cells to a quiescent, nondividing stage (*4*), and/or to promote cell death (*5-8*). In this issue of the Journal, Mehta et al. (*9*) report the effect of a novel vitamin D compound in a murine mammary gland chemoprevention model. The secosteroid hormone known as 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] has been described as a key regulator of serum calcium. In the last two decades, however, it has also been found to have diverse biologic effects in normal and malignant tissues. These responses include the in vitro inhibition of proliferation and induction of differentiation of various cancer cells, such as those from the human hematopoietic system, breast, ovaries, colon, brain, and prostate (*10-17*). Initiation of these genomic responses is through a specific steroid hormone nuclear vitamin D_3 receptor (VDR) acting as a ligand-inducible transcription factor that binds the vitamin D_3 response element contained within the promoter/enhancer region of target genes (*18*).

Despite the intense research that has focused on 1α,25$(OH)_2D_3$ since it was first characterized in 1971 (*19*), the exact mode of action by which it inhibits cancer cells remains largely unknown. In normal tissues not directly involved in calcium regulation, for example, the well-studied system of keratinocytes, exposure of these cells to 1α,25$(OH)_2D_3$ increases the synthesis of transforming growth factor (TGF)-β1 and TGF-β2 (*20*), decreases expression of epidermal growth factor receptors (*21*), and leads to dephosphorylation of the retinoblastoma protein (*22*). In normal prostate cells, it exerts a differentiating effect in combination with testosterone (*23*). At pharmacologically active doses, 1α,25$(OH)_2D_3$ can suppress the immune system (*24-27*) and can enhance monocyte–macrophage differentiation (*28*). Other specific, genomic effects observed in cancer cells exposed to 1α,25$(OH)_2D_3$ include cell cycle arrest in G_1. Many factors can lead to cell cycle arrest, but the cyclin-dependent kinase inhibitors known as $p21^{(waf\ 1)}$ and $p27^{kip\ 1}$ are pivotal to this process; the $p21^{(waf\ 1)}$ gene contains a vitamin D_3 response element within its promotor region (*29*) and expression of the gene increased in response to 1α,25$(OH)_2D_3$. Also, expression of $p27^{(kip\ 1)}$ is markedly induced in certain cancer cell types (e.g., myeloid leukemia and prostate cancer) after their exposure to 1α,25$(OH)_2D_3$ (*17,30-32*).

A major focus of chemoprevention research in the field of vitamin D and cancer has been to synthesize analogues of 1α,25$(OH)_2D_3$ that have prominent antiproliferative effects against cancer cells without resulting in hypercalcemia when they are administered in vivo at pharmacologically active doses. This research has resulted in several analogues that have dramatic antiproliferative behavior, most noticeably analogues with double and triple bonds in the C/D ring and side chain (*33,34*), addition of three to six hexafluoride groups to the end of the side chain (*17,28,35*), or placement of the side chain in the 20-epi configuration (*36-38*). Initial clinical trials are under way; for instance, an ongoing phase I study in the U.K. is examining the effects of these analogues on breast cancer. Studies in vitro have shown that vitamin D_3 analogues can inhibit the clonal proliferation of breast cancer cells at the 10^{-11}-10^{-9} *M* range, with an associated increase in expression of bax and concurrent decrease in bcl-2 expression (*37*). Furthermore, potent hexafluoride analogues can reduce the breast cancer incidence and burden in *N*-nitroso-*N*-methylurea-treated rats (*39*). One area in which the therapeutic potential of vitamin D_3 has been realized is in the treatment of psoriasis, where the topical application of potent analogues, including calcipotriene (Dovonex), controls the disease and does not significantly interfere with serum calcium levels (*40*).

The reason for the increased antiproliferative potency of the analogues is becoming clearer, as illustrated in Fig. 1. Vitamin D_3 analogues usually bind less well to the D-binding protein in the blood and are, therefore, more readily available to enter the cells (*41*). Analogues may also extend the half-life of the VDR (*42*), or they may induce novel VDR conformations (*43*), which may either allow more efficient interactions with vitamin D_3 response elements and/or expand the array of vitamin D_3 response elements that can be activated. In addition, metabolic products of analogues may result in potent intermediates in vivo. For example, compared with the parental analogues, the 24-oxo metabolites have the same in vitro anticancer activities but have fewer effects on calcium levels in sera (*44,45*).

In vivo, VDR forms heterodimers with the retinoid X recep-

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See "Notes" following "References."

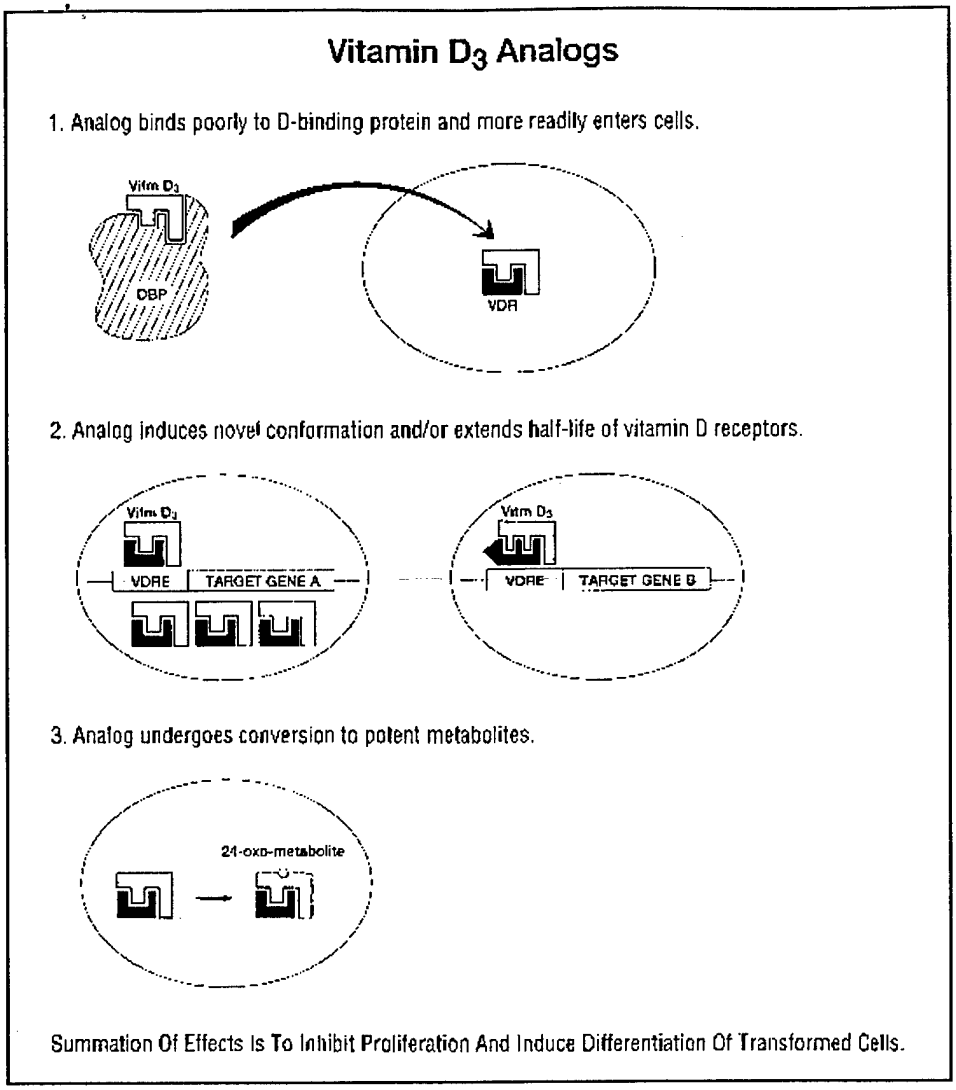

Fig. 1. Mechanism by which vitamin D analogues may have enhanced cellular effects. DBP, D-binding protein; VDR, vitamin D receptor; VDRE, vitamin D response element.

tor (*18*), and the combination of 9-*cis*-retinoic acid and $1\alpha,25(OH)_2D_3$ can synergistically increase expression of a reporter gene construct containing a vitamin D_3 response element within its promoter (*46*). Cooperation between these two receptor pathways has been the basis for combination therapy; we have previously demonstrated (*47,48*) synergistic inhibition of proliferation of human myeloid leukemia cells and MCF-7 breast cancer cells by a potent vitamin D_3 analogue in combination with 9-*cis*-retinoic acid. Thus, combinations of retinoids and vitamin D ligands may be an attractive prospect for control of deregulated cell growth. Also, various steroid hormone receptor enhancer proteins have been identified (*49,50*); by recruiting them in vivo, we may be able to accentuate further the positive therapeutic genomic effects of $1\alpha,25(OH)_2D_3$.

The study by Mehta et al. reported in this issue of the Journal presents an entirely novel class of vitamin D compounds (vitamin D_5). Utilizing a mammary gland lesion model to assess chemoprevention, the authors demonstrated preventive effects in vitro but no significant effect on serum calcium levels in vivo. Thus, the therapeutic index (ratio of its antiproliferative to its calcemic effects) for this compound is sufficiently high to warrant further investigations using other cancer cell types and model systems. The study by Mehta et al. and ongoing fundamental research into the effects of vitamin D compounds on cancer cells are elucidating the molecular effects and highlighting the therapeutic potential of these highly interesting compounds. Several analogues have already been identified that have significant inhibitory effects but that do not induce hypercalcemia; Mehta et al. add another compound to this list. Many of these compounds are potential candidates for clinical investigations.

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Notes

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Analysis of Cytokine Profiles in Patients With Human Papillomavirus-Associated Neoplasms

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Cervical cancer is the second leading cause of cancer mortality in women worldwide, with approximately half a million new cases occurring annually (*1*). More than 90% of cervical cancers and their precursors, so-called cervical intraepithelial neoplasia, contain human papillomavirus (HPV) DNA sequences, and it is now well established that HPV has a major causal role in the development of cervical neoplasia (*2*). It has also been recognized that HPV infection of the cervix and lower genital tract is one of the most common sexually transmitted diseases (*3*). Prevalence rates of HPV infection (based on nucleic acid amplification techniques) can be only crudely estimated because they are influenced by several factors. These rates vary substantially according to the population studied and are highly age dependent. In the United States, HPV prevalence ranges from 25%-30% in asymptomatic women aged 15-25 years to approximately 5% in women aged 33-55 years, an age group in which most cervical cancers are detected (*2,4*). In contrast, there are approximately 15 000 incident cases of cervical cancers in the United States annually (*5*). These observations have led investigators to postulate that other factors, in addition to HPV, play a role in cervical carcinogenesis.

Several lines of evidence suggest that cell-mediated immune responses are important in controlling both HPV infections and HPV-associated neoplasms [for review, *see* (*6*)]. First, the prevalence of HPV-related diseases (infections and neoplasms) is increased in transplant recipients and in patients infected with human immunodeficiency virus (HIV); both types of patients are known to have impaired cell-mediated immunity (*7,8*). Second, studies on animals have demonstrated that immunized animals are protected from papillomavirus infection and from the development of neoplasia. Immunization also facilitates the regression of existing lesions (*9-11*). Third, infiltrating $CD4^+$ (T-helper cells) and $CD8^+$ (cytotoxic/suppressor T cells) T cells have been observed in spontaneously regressing warts. Fourth, warts seen in patients who are on immunosuppressive therapy often disappear when this treatment is discontinued.

Cell-mediated immunity is regulated by cytokines that are secreted by T helper cells. In general, T-helper cells can be classified as Th1 and/or Th2 cells on the basis of the different types of cytokines they secrete. Th1 cells secrete interleukin (IL) 2 (IL-2) and interferon gamma (IFN γ). Th2 cells produce IL-4, IL-5, IL-10, and IL-13. The Th1 lymphocytes are the most important effector cells in inflammatory reactions associated with vigorous delayed-type hypersensitivity but low antibody production, as occurs in contact dermatitis and in viral or intracellular bacterial infections [for review, *see* (*12,13*)]. The functional phenotype of most Th2 cells may account for both the persistent production of certain antibody isotypes, particularly immunoglobulin G_1 and immunoglobulin E, and the eosinophilia observed in human helminthic infections and allergic disorders. Although the Th1 and Th2 phenotypes were first described in mice, clones of Th1 and Th2 cells have also been isolated from humans. For example, most $CD4^+$ T cells that infiltrate the thyroid gland in patients with autoimmune thyroid diseases are of the Th1 type, since they can be induced to produce IFN γ but not IL-4. In contrast, the great majority of allergen-specific T-cell clones derived from patients with allergic disorders express the Th2 phenotype, as evidenced by their production of IL-4 and IL-5 and their limited, if any, production of IFN γ (*13*). Lymphocyte-mediated protection from viral infections as well as control of tumors is thought to be mediated by Th1 cytokine responses and impaired by Th2 cytokine responses. The IL-2- and IFN γ-producing Th1 response is likely to be the major component that contributes to the development of cell-mediated immunity against HPV infections and HPV-associated neoplasms.

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In this issue of the Journal, Clerici et al. (*14*) report on the cytokine profiles of stimulated peripheral blood mononuclear cells (PBMCs) in women with precursors of cervical cancer (grade III cervical intraepithelial neoplasia) and HPV infection. Women with HPV infection limited to the cervix were compared with women with more extensive HPV infection that involved other sites of the lower genital tract. These investigators demonstrate that IL-2 production by PBMCs, in response to stimulation with soluble antigen or alloantigens (i.e., major histocompatibility complex [MHC] antigens), was reduced in the group of patients with extensive HPV infection compared with the group of patients with localized disease and with healthy control subjects. Furthermore, IL-4 and IL-10 production in response to mitogen stimulation was elevated in the group with extensive disease compared with the response seen in the group with localized disease or healthy control subjects.

The same group of investigators (*15*) made similar observations in an earlier study performed on asymptomatic, HIV-seropositive individuals. A sequential and progressive loss of Th1 function was observed; it was manifested first by the loss of the IL-2 response to recall antigens, then by the IL-2 response to alloantigens, and finally by the IL-2 response to phytohemagglutinin (PHA). Furthermore, it was reported (*16*) that these defects were associated with, or predictive of, a variety of acquired immunodeficiency syndrome (AIDS)-related events. Individuals who were unresponsive to all three stimuli (recall antigens, alloantigens, and PHA) exhibited a more rapid decline in $CD4^+$ T-cell numbers, a higher frequency of bacterial and opportunistic infections, and a greater likelihood for their disease to progress rapidly to AIDS. A similar decrease in the Th1 response with predominance of the Th2 cytokine profile has also been observed in patients with such neoplasms as Sézary syndrome (*17*), Hodgkin's disease (*18*), bronchogenic carcinoma (*19,20*), renal cell carcinoma (*21,22*), lymphomas (*23*), glioma (*24*), and melanoma (*25,26*). These observations clearly demonstrate that the Th2 phenotype is associated with persistence of viral infection and development of neoplasms.

Although the study by Clerici et al. presented in this issue of the Journal did not use an HPV-specific peptide as a recall antigen, HPV antigen-specific assays were performed in a study by Tsukui et al. (*27*) and showed similar findings. Tsukui et al. demonstrated that Th1 cytokine production, specifically IL-2 production, in response to HPV-16 E6 and E7 peptides is diminished in patients with high-grade cervical intraepithelial lesions and cervical cancer compared with that in cytologically normal women (*27*). These findings are consistent with the hypothesis that the production of cytokines that enhance potentially protective cell-mediated immunity is defective in women with extensive HPV infection and that progression to cervical cancer precursor lesions may be associated with a Th1-to-Th2 switch in immunoregulatory cytokine production. At present, it is unknown whether this Th1-to-Th2 shift leads to the development of neoplasia by subverting immune surveillance mechanisms or whether the shift is a secondary effect induced by the tumor cells or by the persistent viral infection itself. One argument against the hypothesis that the shift from a Th1 to a Th2 response is the cause of the progression to neoplasia is that patients with allergic disorders, who in general have high Th2-type responses, do not appear to have higher rates of viral infections or cancer (*28*). Furthermore, HPV-infected epithelial cells have been shown to modify cytokine profiles. Lymphokine secretion by HPV-immortalized and cervical carcinoma cell lines has been demonstrated in culture (*29*). In addition, constitutive release of IL-6 by HPV-16-harboring keratinocytes (*30*) and enhanced IL-6 gene expression in invasive cervical carcinoma have been reported (*31*). These findings suggest that HPV-infected keratinocytes can modify local immune responses and possibly can influence systemic cytokine profiles. Accordingly, it will be important to conduct prospective, longitudinal studies to determine whether the shift of cytokine profiles from Th1 to Th2 is a causal factor or an epiphenomenon.

It has also been shown that the pattern of cytokine profiles can be modified in vitro and in vivo. For example, IL-12 or IFN γ can decrease Th2 clones, and IL-10 can decrease Th1 clones. IL-12 has been shown to restore IFN γ production by PBMCs from HIV-seropositive patients in whom IFN γ production by PBMCs was reduced or lost (*32*). In an in vivo murine model, treatment with recombinant murine IL-12 cured most BALB/c mice infected with *Leishmania* (*33*). Cure was associated with a markedly depressed production of IL-4 by lymph node cells cultured with antigen or mitogen, whereas IFN γ production was preserved or increased. Thus, murine IL-12 prevents deleterious Th2 T-cell responses and promotes curative Th1 responses in an IFN γ-dependent fashion during murine leishmaniasis. These findings suggest that treatment with cytokines may have potential in augmenting the diminished immunologic functions associated with progression of those diseases influenced by inappropriate cytokine production.

In summary, the study by Clerici and colleagues (*14*) adds to our growing understanding of the mechanisms by which the immune system mediates the behavior of HPV-associated neoplasms. Further studies aimed at elucidating how cytokine production is regulated in response to HPV infection and what the role of cytokines is in the critical step between infection and neoplasia hold promise for developing novel approaches to the therapy of HPV infection and cervical cancer.

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Ligands for peroxisome proliferator-activated receptorγ and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice

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ABSTRACT **Induction of differentiation and apoptosis in cancer cells through ligands of nuclear hormone receptors (NHRs) is a novel and promising approach to cancer therapy. All-*trans*-retinoic acid (ATRA), an RA receptor-specific NHR ligand, is now used for selective cancers. The NHR, peroxisome proliferator-activated receptor γ (PPARγ) is expressed in breast cancer cells. Activation of PPARγ through a synthetic ligand, troglitazone (TGZ), and other PPARγ-activators cause inhibition of proliferation and lipid accumulation in cultured breast cancer cells. TGZ (10^{-5} M, 4 days) reversibly inhibits clonal growth of MCF7 breast cancer cells and the combination of TGZ (10^{-5} M) and ATRA (10^{-6} M, 4 days) synergistically and irreversibly inhibits growth and induces apoptosis of MCF7 cells, associated with a dramatic decrease of their bcl-2 protein levels. Similar effects are noted with *in vitro* cultured breast cancer tissues from patients, but not with normal breast epithelial cells. The observed apoptosis mediated by TGZ and ATRA may be related to the striking down-regulation of bcl-2, because forced over-expression of bcl-2 in MCF7 cells cultured with TGZ and ATRA blocks their cell death. TGZ significantly inhibits MCF7 tumor growth in triple immunodeficient mice. Combined administration of TGZ and ATRA causes prominent apoptosis and fibrosis of these tumors without toxic effects on the mice. Taken together, this combination may provide a novel, nontoxic and selective therapy for human breast cancers.**

The high prevalence of breast cancer and the limited therapeutic possibilities provide a strong stimulus for identification of new, selective molecular targets for anticancer therapy. Cancers are associated with dysregulation of differentiation and apoptosis. Induction of these processes through ligands of nuclear hormone receptors (NHRs) is a recent approach to cancer therapy, particularly, in the use of RAs for treatment of acute promyelocytic leukemia (1), early lesions of head and neck cancer (2), squamous cell carcinoma of the cervix (3), and skin cancer (4). The actions of retinoids are mediated by RA receptors (RARs) and retinoic X receptors (RXR) both of which are expressed in breast cancer cells. The RARs and RXRs bind to specific RA-responsive elements and regulate transcription of target genes in a ligand-dependent manner (5–6). Retinoids are highly effective in preventing mammary carcinogenesis in rodents (7). The RAR-specific ligand, all-*trans*-RA (ATRA) selectively inhibits growth of human estrogen receptor (ER)-positive breast cancer cells (8–10), and these cells express higher levels of RARα mRNA than ER-negative lines (10–11). Furthermore, activation of gene transcription by RARα appears to be required for inhibition of growth in human ER-positive breast cancer cells by retinoids (12). However, inhibition of growth of breast cancer cells through retinoids is usually reversible with removal of the ligand (13).

The peroxisome proliferator-activated receptor γ (PPARγ), which also belongs to the NHR superfamily, has an important role in differentiation of adipocytes and in fat metabolism (14-15). Epidemiological data suggest that the incidence of breast cancer is related to consumption of a diet high in fat (16–17). Breast cancer cells have significant lipogenic capacity, and inhibition of fat metabolism in these cells is associated with inhibition of growth and apoptosis (18). Recent data showed that human breast cancer cell lines as well as primary and metastatic breast adenocarcinomas expressed PPARγ and ligand activation of PPARγ caused inhibition of proliferation and extensive lipid accumulation in cultured breast cancer cell lines (19–20).

Various NHRs can interact with each other by suppression or activation of their target genes. For example, RXRs activated (21) and RARs suppressed the induction of PPARγ (22) in normal fat cells. Our previously data showed crosstalk between the NHRs; for example, ligand-activation of receptors for RAR/RXR and vitamin D_3 resulted in a synergistic decreased proliferation and induction of apoptosis of leukemia cell lines (23–24). In this investigation, we analyzed the ability of a synthetic-specific ligand of PPARγ, the antidiabetic drug troglitazone (TGZ; ref. 25) as well as several other PPARγ ligands either alone or in combination with ATRA to affect the growth, differentiation and apoptosis of breast cancer cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Lines and Samples. All cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained according to their recommendations. Fresh breast cancer and adjacent normal breast tissue from three individuals were surgically obtained after their informed consent.

Ligands. TGZ {5[4(3, 4-Dihydro-6-hydroxy-2, 5, 7, 8-tetramethyl-2H-1-benzopyran-2-yl)methoxy]phenyl[methyl]-2,4-thiazolidinedione} (generous gift of W. Johnson and A. Salteil. Park-Davis/Warner-Lambert, Ann Arbor, MI), and ATRA (Sigma) were dissolved in 100% ethanol. The 15-deoxy-Δ12, 14-prostaglandin J_2 (15d-PGJ_2, Calbiochem, La Jolla, CA) and indomethacin (Sigma) were dissolved in dimethyl sulfoxide.

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Abbreviations: NHR, nuclear hormone receptor; ATRA, all-*trans*-retinoic acid; PPARγ, peroxisome proliferator-activated receptor γ; TGZ, troglitazone; RAR, RA receptor; ER, estrogen receptor; RXR, retinoic X receptors; AP, alkaline phosphatase; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling.

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Each of the diluants either alone or in combination at concentrations of 10^{-5} M or less had no significant effect on breast cancer cell lines.

Clonogenic Assay in Soft Agar. Effect of NHR ligands on clonogenic growth of breast cancer cells was determined by dose-response studies in soft agar as described (26).

Measurement of Cellular Content of Proteins and Lipid Accumulation. Expression of specific proteins was detected by Western blot, flow cytometry (24), or by immunohistochemistry of either cytospined MCF7 cells grown *in vitro* or freshly fixed in formalin and embedded in paraffin (24, 26). Western blotting, immunodetection, and stripping of membranes were performed by using standard methods (60 μg of protein per lane), as recommended by the supplier of the Enhanced Chemiluminescence Detection System (Amersham). Densitometric measurements were done by using "UVP gel analysis suite." Anti-PPARγ polyclonal antibody (516555, 1:2000, Calbiochem), anti-bcl-2 murine mAb, (clone 124, 1:50, Dako), anti-bax polyclonal antibody (N20, 1:50, Santa Cruz, CA); anti CD36 mAb (1:100, Immunotech, Luminy, France), anti-E-cadherin mAb (1:1500, Transduction Laboratories, Lexington, KY), and anti-β-casein mAb (MAS 447 1:100, Harlan Sera-Lab, Sussex, England) were used. Expression of proteins in MCF7 cells stained by immunohistochemistry were measured by the total intensity score (0–300), which was calculated as the sum of the products of each intensity score (0–3) and their corresponding percentages from 300 cells. Normal IgG was substituted for specific antibody for each experiment as a negative control. Measurement of lipid accumulation was performed by staining of cells with Oil Red O (26). Human breast adipocytes were used as positive control.

Measurement of Apoptosis. DNA strand breaks were identified by terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) technique using *in situ* Cell Death Detection Kit, used either fluorescein or alkaline phosphatase (AP) as recommended by the supplier (Boehringer Mannheim).

Transfection of MCF7 Cells with bcl-2 Vector. MCF7 cells were transfected with a cytomegalovirus-bcl-2 expression plasmid (generous gift from J. Reed, The Burnham Institute, La Jolla, CA) or empty vector (pcDNA3) using Superfect (Qiagen). As a marker for transfection, a plasmid coding for green fluorescent protein (EGFP, CLONTECH) driven by the cyclin A1 promoter (C. Müller and H. P. Koeffler, unpublished data) was cotransfected at a ratio of 1:4. The green fluorescent protein-expressing cells were selected using a FACStar (Becton Dickinson) flow cytometric sorter.

Northern Blot Analysis. Total RNA was extracted by using Trizol (GIBCO/BRL). Blots (30 μg of total RNA) were hybridized with ^{32}P-labeled CCAT-Enhancer Binding Protein (5.0-kb *Bam*HI genomic fragment), as well as aP2, lipoprotein lipase, and adipsin (cDNA). Blots were rehybridized with β-actin probe as control.

***In Vivo* Murine Cancer Model.** Female triple-immunodeficient BNX nude mice (Harlan–Sprague–Dawley) at 8 weeks of age were whole body irradiated (300 rads) and 5×10^6 of MCF7 cells in 0.1 ml of Matrigel (Collaborative Biomedical Products, Bedford, MA) were bilaterally injected s.c. into the trunk of 20 mice, forming two tumors/mouse. Treatment was started on the day after the injection of these human breast cancer cells and finished after 9 weeks. Cohorts (5 mice/group) received diluant only (control group), troglitazone (1,000 mg/kg/day, orally by gavage), ATRA (7.5 mg/kg/day, i.p.), or both ligands. After 9 weeks, bloods were collected for chemistry and hematological analyses. Tumors, livers, lungs, spleens, and kidneys were fixed and stained for histological analyses. All animal experiments were in compliance with National Institutes of Health guidelines.

Statistical Analysis. All numerical data were expressed as the average of the values obtained ± SD. Statistical significance of differences between tumors in mice was analyzed by nonparametric Mann–Whitney *U* test using STAT VIEW software (Abacus Concept, Berkeley CA). For all other experiments, significance was determined by conducting a paired Student's *t* test.

RESULTS

Expression of PPARγ Protein. Breast adenocarcinoma cells from patients (Fig. 1) expressed high levels of PPARγ protein as seen by immunohistochemistry. In contrast, normal breast epithelial cells from individuals with breast cancer expressed low levels of PPARγ protein (Fig. 1). The rank order of expression of PPARγ in the breast cancer cell lines was: BT474>MCF7>T47D>MDA-MB-231, as measured by Western blot (Fig. 2).

Effect of Ligands on Proliferation of Breast Cancer Cells *in Vitro*. Sensitivity of breast cancer cell lines to inhibition of clonal growth by TGZ is shown on Fig. 3. The MCF7 cells were the most sensitive to the inhibitory effects of TGZ (MCF7>MDA-MB-231>BT474>T47D) with an effective dose (EC_{50}) of 10^{-7} M, resulting in the inhibition of 50% clonal growth (Fig. 3). Dose-response studies with ATRA showed an EC_{50} of 4×10^{-9} M for MCF7 cells, and the combination of various concentrations of TGZ together with 10^{-9} M ATRA enhanced this inhibition (Fig. 3). Pulse-exposure of MCF7 cells for 4 days to either TGZ (10^{-5} M) or ATRA (10^{-6} M), washing extensively and culturing in agar, resulted in 6% and 32% decrease, respectively in clonogenic growth (Fig. 4). Thus, growth inhibition by either TGZ or ATRA was partially reversible. In contrast, a 4-day pulse-exposure to both TGZ (10^{-5} M) and ATRA (10^{-6} M) irreversibly inhibited 80% clonogenic growth compared with untreated MCF7 cells (Fig. 4). The 15d-PGJ_2, natural ligand for PPARγ, and indomethocin, also a PPARγ ligand (10^{-10}-10^{-5} M) inhibited clonal growth of MCF7 cells; and the rank order of inhibition was 15d-PGJ_2>TGZ>indomethacin (data not shown), and this inhibition was reversible (Fig. 4). However, the combination of ligands for PPARγ and RAR induced irreversible inhibition of clonal proliferation (Fig. 4).

Analysis of Apoptosis in MCF7 Cells. ATRA (10^{-6} M, 4 days) did not induce apoptosis of MCF7 cells (Table 1). TGZ (10^{-5} M, 4 days) increased only slightly the percentage of apoptotic cells (13 ± 8%) compared with untreated cells (8 ± 3%). In contrast, the combination of both ligands significantly increased the number of MCF7 cells undergoing apoptosis (41 ± 4%, $P < 0.05$, Table 1). In addition, either 15d-PGJ_2 or

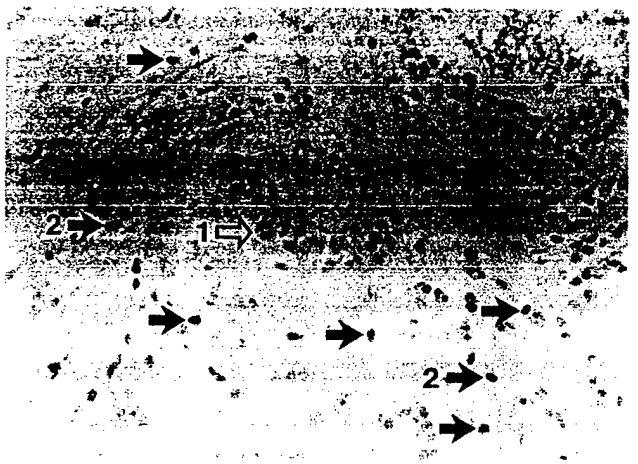

FIG. 1. Expression of PPARγ protein in infiltrating ductal breast adenocarcinoma. Benign breast ducts show low immunoreactivity (arrow 1), whereas infiltrating carcinoma cells are strongly positive (arrow 2).

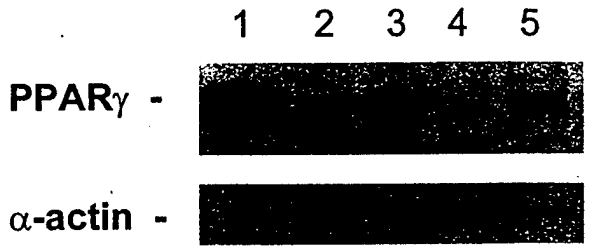

FIG. 2. Expression of PPARγ protein in human breast cancer cell lines. Lane 1, human breast adipocytes (positive control); lane 2, T47D cells; lane 3, MCF7 cells; lane 4, MDA-MB-231 cells; lane 5, BT474 cells. The α-actin is control for the amount of loaded protein.

indomethacin (10^{-5} M) combined with ATRA (10^{-6} M) for 4 days induced apoptosis (Table 1).

The same MCF7 cell cultures that were examined for apoptosis also were examined for apoptosis-related proteins. The TGZ did not change the level of bcl-2 protein (Fig. 5*A*) compared with untreated cells. ATRA decreased the level of bcl-2 to 44% of untreated cells ($P < 0.01$), and the combination of both ligands decreased bcl-2 protein to nearly undetectable levels (<5% of control cells) (Fig. 5*A*). In contrast, levels of bax after exposure of MCF7 cells to either TGZ alone or TGZ and ATRA changed little, and ATRA alone increased levels by ≈20% compared with untreated MCF7 cells (Fig. 5*A*). As compared with untreated cells, levels of PPARγ protein in MCF7 cells slightly decreased by culturing with either TGZ (83%), ATRA (48%), or a combination of ATRA and TGZ (66%).

Effect of NHR Ligands on bcl-2-Transfected MCF7 Cells. Because the combination of TGZ and ATRA mediated both a profound decrease in bcl-2 levels and a marked increase in apoptosis, we hypothesized that the two events were linked. Therefore, the experiments were repeated in MCF7 cells transfected with bcl-2 expression vector. More than 70% of the bcl-2 transfected MCF7 cells over-expressed bcl-2 protein as measured by immunochistochemistry (data not shown), and bcl-2 protein expression was >10-fold higher in bcl-2 transfected MCF7 cells compared with MCF7 cells transfected with empty vector as measured by Western blot (Fig. 5*B*). The bcl-2-transfected MCF7 breast cancer cells no longer underwent apoptosis after exposure to TGZ and ATRA; under the same conditions, those transfected with empty vector did undergo apoptosis (Table 1).

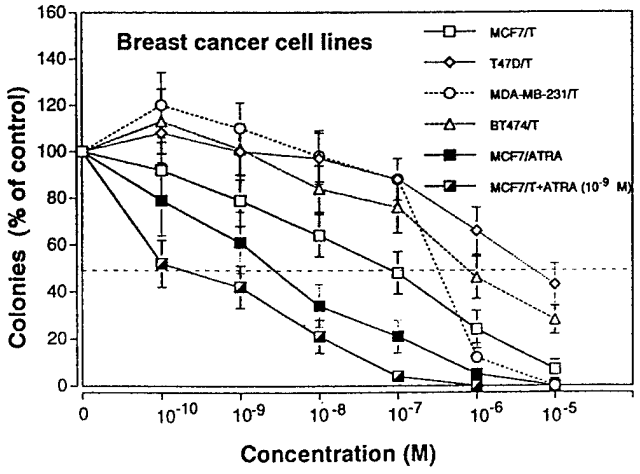

FIG. 3. Dose-response effect of TGZ and/or ATRA on clonal proliferation of breast cancer cell lines Results are expressed as the mean percentage of colonies in control plates containing no ligand. Each point represents mean ± SD of three independent experiments with triplicate dishes.

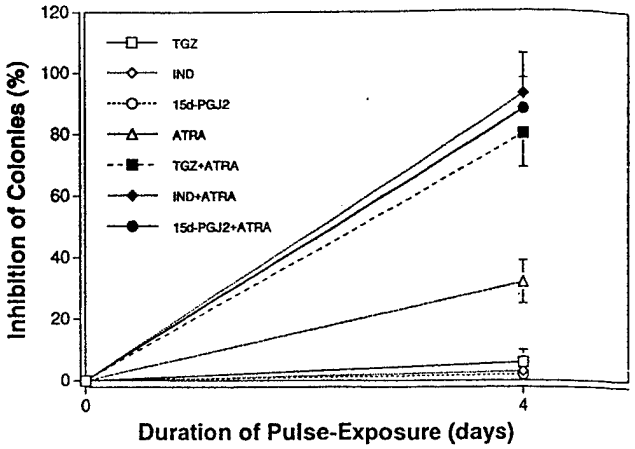

FIG. 4. Pulse-exposure of MCF7 cells to TGZ (10^{-5} M), indomethacin (IND) (10^{-5} M), 15d-PGJ_2 (10^{-5} M), and/or ATRA(10^{-6} M). Results are expressed as the mean percentage of control plates containing no ligand. Each point represents a mean ± SD of three independent experiments with triplicate dishes.

Effect of NHR Ligands on Expression of Lipid and Differentiation Markers in MCF-7 Cells. Untreated MCF7 cells were almost negative for lipid accumulation, as measured by staining with Oil-red O (Table 2), but these cells expressed the lipid metabolism-associated CD36 protein, as demonstrated by immunohistochemistry (Table 2) and flow cytometry (data not shown). After exposure of MCF7 cells to TGZ (10^{-5} M, 4 days), ≈87% MCF7 cells stained strongly for lipid (Table 2), and their expression of CD36 protein increased compared with untreated MCF7 cells (Table 2). Exposure to ATRA alone decreased CD36 expression without a change in lipid accumulation compared with untreated cells (Table 2). In contrast, the combination of both ATRA (10^{-6} M, 4 days) and TGZ (10^{-5} M, 4 days) dramatically decreased lipid accumulation and CD36 expression in MCF7 cells compared with TGZ-treated MCF7 cells (Table 2). Although TGZ induced lipid accumulation in MCF7 cells, these cells did not change their pattern of differentiation either to adipocytes as measured by expression of adipocyte-associated transcripts for C/EBPα, aP2, lipoprotein lipase, or adispin (data not shown), or to more differentiated breast cancer cells as measured by β-casein and E-cadherin (Fig. 5*A*).

***In Vitro* Effect of NHR Ligands on Breast Adenocarcinoma Cells and Normal Breast Epithelial Cells from Patients.** Both normal and breast adenocarcinoma tissues from three patients cultured for 4 days with either TGZ (10^{-5} M) or ATRA (10^{-6}

Table 1. Apoptosis in nontransfected and bcl-2-transfected MCF7 cells

	Apoptotic cells, %		
Ligands	non-transfected	bcl-2 expression vector	empty vector
(−)	8 ± 3	10 ± 3	12 ± 4
ATRA	7 ± 5	11 ± 5	± 6
TGZ	13 ± 8	11 ± 6	± 9
TGZ + ATRA	41 ± 4	10 ± 4	58 ± 8
IND	12 ± 4	ND	ND
IND + ATRA	44 ± 6	ND	ND
15dPGJ$_2$	18 ± 7	ND	ND
15dPGJ$_2$ + ATRA	46 ± 4	ND	ND

Apoptosis determined by TUNEL assay. ND, not done; IND, indomethacin. Cells exposed 4 days to ATRA (10^{-6} M), TGZ (10^{-5} M), IND (10^{-5} M), and 15dPGJ$_2$ (10^{-5} M). Results represent the mean ± SD of three experiments.

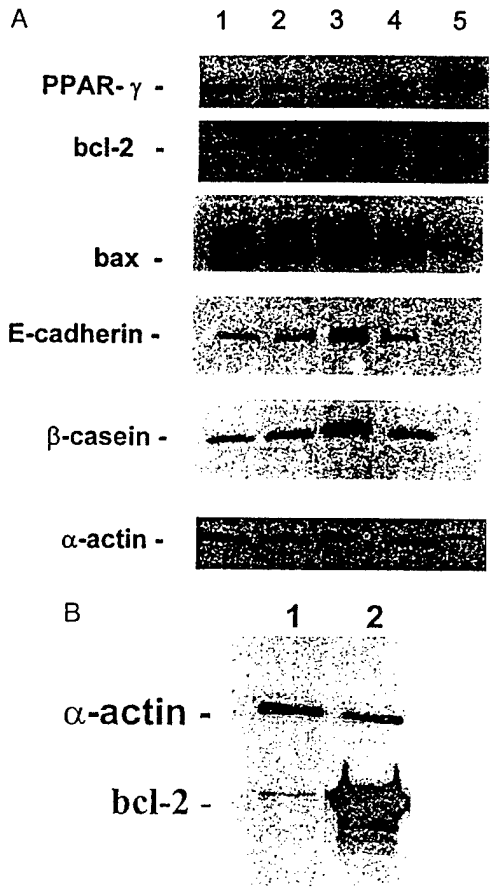

FIG. 5. (*A*) Expression of PPARγ, bcl-2, bax, E-cadherin, and β-casein in MCF7 cells after their incubation with ligands for 4 days as measured by Western blot. Lane 1, control (vehicle alone); lane 2, TGZ (10^{-5} M); lane 3, ATRA (10^{-6} M); lane 4, TGZ (10^{-5} M) +ATRA (10^{-6} M); lane 5, human breast adipocytes (positive control). (*B*) Expression of bcl-2 protein in bcl-2 transfected MCF7 cells as measured by Western blot (20 μg of protein per lane).

M) showed no changes either in morphology or apoptosis. However, the combination of both caused massive apoptosis (>80% of cells as measured by TUNEL) in each cancer sample but not in the accompanied normal breast epithelial cells (<10% apoptotic cells) (data not shown).

Antitumor Effect *in Vivo*. The TGZ visibly inhibited the tumor growth of MCF7 cells in triple-immunodeficient mice as measured by tumor size ($P < 0.003$) (Figs. 6) as well as tumor weights resected at autopsy ($P < 0.006$) (data not shown) compared with those of diluant-treated control animals. Combination of ATRA and TGZ or ATRA alone also significantly inhibited the size ($P < 0.001$) (Fig. 6) and weights ($P < 0.001$) (data not shown) of the tumors. Histological analysis of MCF7 tumors from untreated mice revealed poorly differentiated infiltrating adenocarcinomas (data not shown) almost without apoptotic changes (Fig. 7*A*). Mice treated with either troglitazone or ATRA showed some apoptosis as measured by morphology (data not shown) and TUNEL assay (Fig. 7 *B* and *C*). However, in mice treated with the combination of both troglitazone and ATRA, almost all of the MCF7 tumor cells were either apoptotic or necrotic as measured by morphology (data not shown) or by TUNEL assay (Fig. 7*D*), and extensive fibrosis of the tumors was observed (data not shown).

Table 1. Expression of lipid-related markers in MCF7 cells cultured with TGZ (10^{-5} M), ATRA (10^{-6} M), or combination of both for 4 days

Ligands cells	Oil-red O positive cells, TIS units	CD36-positive, %
(−)	4 ± 3	64 ± 12
TGZ	280 ± 11	90 ± 14
ATRA	2 ± 1	36 ± 8
TGZ + ATRA	42 ± 14	18 ± 7

TIS units defined in *Materials and Methods*. Results represent the mean ± SD of three experiments.

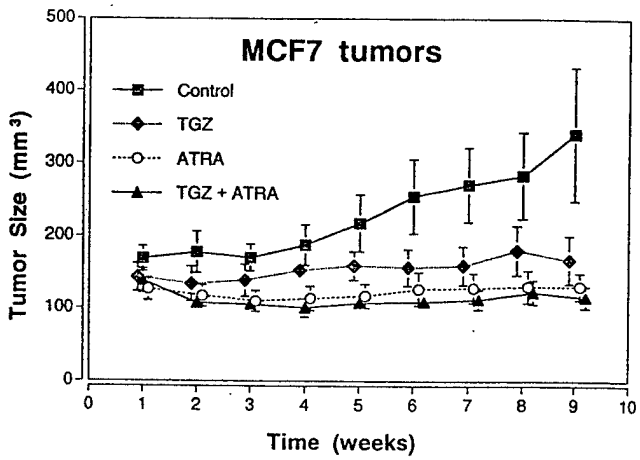

FIG. 6. Effect of TGZ and ATRA, either alone or in combination on the size of MCF7 tumors in BNX triple immunodeficient mice. Results represent the mean ± SD of 10 tumors.

No significant difference in either the mean weights, histology of internal organs, mean blood chemistries including liver parameters as well as hematopoietic parameters was found between diluant treated mice and those that received 9 weeks of treatment (data not shown), except for a decrease of the cholesterol level in the experimental groups that received both troglitazone and ATRA compared with the untreated mice (71 ± 21 mg/dl and 87 ± 13 mg/dl, respectively).

DISCUSSION

The PPARγ functions as an important regulator of lipid metabolism, and it is a key mediator of lipid storage (27–28). Our data show that human breast cancer cell lines and fresh breast adenocarcinomas express PPARγ protein, which is consistent with data from other groups (19–20). Furthermore, our histologic data indicated that, in contrast to breast cancer cells, the normal human breast epithelial cells lining the mammary ducts expressed a low level of PPARγ protein as seen in three individuals with breast cancer. Therefore, we hypothesized that prominent expression of PPARγ protein

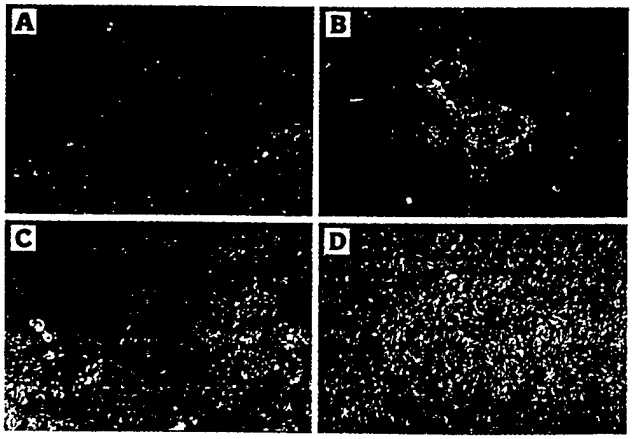

FIG. 7. Apoptotic cells in MCF7 tumors in BNX triple immunodeficient mice as measured by TUNEL assay. control mice (*A*); treated with TGZ (*B*); ATRA (*C*); combination of both ligands (*D*).

might be a marker for breast cancer cells; however, this hypothesis requires analysis of more normal and malignant breast tissues. In contrast to our data, another group (20) found strong positivity for PPARγ protein in the normal epithelial cells lining the mammary ducts from one patient. The explanation for these differences in results are unclear but may reflected distinct stages of normal mammary ducts (lactigenic or nonlactigenic period). The normal breast tissues of our patients were nonlactigenic.

Clonal growth of each of the breast cancer cell lines were inhibited by TGZ, a synthetic ligand of PPARγ. However, the rank order of their sensitivity to TGZ was not the same as their level of PPARγ protein expression. The BT474 cells, which expressed the highest level of PPARγ protein, were relatively insensitive to the inhibitory effect of TGZ. Our data are in agreement with those from another group which showed that a breast cancer cell line with a high level of PPARγ protein expression had a minor response to TGZ (20).

The ER-positive MCF7 cells were the most sensitive of the breast cancer cell lines to the reversible growth inhibitory action of TGZ; and these cells, after culture with TGZ, also had an increased accumulation of fat and an up-regulation of expression of CD36 protein, which is associated with active lipid metabolism and storage (20–30). However, these epithelial cancer cells did not cross-differentiate to adipocytes because TGZ did not induce either increased CCAAT-enhancer binding protein α expression (key transcriptional regulator of terminal adipocyte differentiation) or markers of terminal adipocyte differentiation (aP2, lipoprotein lipase, and adipsin). These data are in agreement with those from Mueller *et al.* 1998 (20). In addition, our data showed that TGZ alone had no significant effect on either the levels of either PPARγ protein, the level of apoptosis, or the induction of differentiation as measured by expression of β-casein and E-cadherin.

The RAR-specific ligand, ATRA reversibly inhibited clonal growth of MCF7 cells and decreased the expression of PPARγ and CD36 proteins. Our data are congruent with observations from another group that showed that a RAR-specific ligand inhibited fat metabolism by suppressing induction of PPARγ (22). Moreover, our studies showed that ATRA slightly increased expression of the apoptosis-related bax protein and decreased the bcl-2 protein level; however, it did not induce apoptosis of the MCF7 cells. In mark contrast, the combination of ATRA and TGZ *in vitro* irreversibly inhibited growth and induced prominent apoptosis of both MCF7 and fresh breast cancer cells but not normal breast epithelial cells. Also, additional ligands for PPARγ were examined: 15d-PGJ_2 and indomethacin (31). Both reversibly suppressed proliferation of MCF7, and each in combination with ATRA caused an irreversible growth inhibition and mark apoptosis of MCF7 cells (Table 2). This effect was associated with a prominent decrease in the expression of bcl-2 and CD36 proteins, as well as a decrease of fat accumulation as compared with TGZ alone. Also, 9-*cis*-RA in combination with TGZ had the same effect as ATRA (data not shown). Interestingly, the combination of a RXR-specific ligand, LG10068 with TGZ did not caused either down-regulation of bcl-2 or apoptosis of MCF7 cells (data not shown), suggesting that activation of RAR may be essential for decreased level of bcl-2 protein and induction of apoptosis.

Forced over-expression of bcl-2 in MCF7 cells completely abolished cell death induced by the combination of a PPARγ ligand and ATRA, suggesting that their induction of apoptosis was linked to a decreased bcl-2 expression. Over-expression of bcl-2 inhibited cell death at confluence or in conditions of serum deprivation in breast cancer cells cultured with very low concentrations of fetal bovine serum (32). Also, alterations in activation of the ER regulate bcl-2 expression in MCF7 cells (33–34) and consequently apoptosis (35). Our data showed that the ER-negative (MDA-MB-231) (data not shown) as well as ER-positive (MCF7) breast cancer cell lines were growth inhibited by the combination of TGZ and ATRA. Therefore, their effects may be independent of estrogen and its receptor. On the other hand, recent data suggest that the PPARγ ligand antagonizes the activation of the AP-1-, nuclear regulatory factor-kappaβ, and the signal transducers and activators of transcription-pathways (36–37) in monocytic cells. Likewise, the AP-1 transcriptional factor can be inhibited by ATRA in MCF7 cells (38), and the blocking of AP-1 by ATRA may be responsible for the antitumor-promoting activity of ATRA (39). Data have shown that blocking of AP-1 activity can be associated with the induction of apoptosis in some types of cancer cells (40). Further studies are needed to examine the combined effect of TGZ and ATRA on the AP-1-, NFKβ- and STAT-pathways.

Our *in vivo* data strongly supported our *in vitro* data. Histological analyses of MCF7 tumors from control mice showed typical breast adenocarcinoma. In contrast, treatment with TGZ plus ATRA markedly inhibited growth of MCF7 tumors associated with down-regulation of bcl-2 (data not shown), with striking apoptosis and fibrosis of these tumors. In fact, the residual mass was almost entirely fibrotic tissue. Mice receiving this combination did not change their weights and had no side effects as measured by extensive blood cell counts, serum chemistries including liver function tests as well as gross-autopsy and histological analyses of the major organs (data not shown). Taken together, the combination of ligands for PPARγ and RAR inhibited growth and induced apoptosis of breast cancer cells *in vitro* and *in vivo*; this combination may provide nontoxic and selective therapy for human breast cancers. The finding that over-expression of bcl-2 inhibited NHR ligands induced apoptosis, suggests a causal relationship between down-regulation of bcl-2 by ligands for PPARγ and RAR and subsequent apoptosis.

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Vitamin D_3 Analogs and Their 24-Oxo Metabolites Equally Inhibit Clonal Proliferation of a Variety of Cancer Cells but Have Differing Molecular Effects

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Abstract The seco-steroid hormone, 1α,25 dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) binds to a specific nuclear receptor that acts as a ligand-inducible transcription factor. The resulting genomic effects include partial arrest in G_0/G_1 of the cell cycle and induction of differentiation; these effects have been observed in various types of cancer. Recently, we produced enzymatically the natural 24-oxo metabolites of 1α,25$(OH)_2D_3$ and two of its potent synthetic analogs (1α,25-$(OH)_2$-16-ene-D_3 and 1α,25-$(OH)_2$-20-*epi*-D_3) using a rat kidney perfusion system. We have found that the 24-oxo metabolites of both 1α,25$(OH)_2D_3$ and its analogs have either the same or greater antiproliferative activity against various cancer cells as their parental compounds. Notably, two cell lines (DU-145 (prostate cancer) and MDA-MB-436 [breast cancer]) that were extremely resistant to the antiproliferative effects of vitamin D_3 analogs displayed greater sensitivity towards the 24-oxo metabolite of the vitamin D_3 analog. Similarly, the 24-oxo metabolites had the capacity to induce differentiation and apoptosis and to diminish the proportion of cells in S phase. Most interestingly, while the analog 1α,25$(OH)_2$-20-*epi*-D_3 induced expression of BRCA1 in MCF-7 breast cancer cells; its 24-oxo metabolite dramatically suppressed BRAC1 expression. Thus, we have shown for the first time that the various biological activities produced by the hormone 1α,25$(OH)_2D_3$ and some of its analogs may represent a combination of actions by the hormone 1α,25$(OH)_2D_3$ and its natural 24-oxo metabolites. J. Cell. Biochem. 66:413–425, 1997.

Key words: vitamin D_3 analogs; 24-oxo metabolites; growth inhibition; differentiation; apoptosis

A common goal of cancer therapy is restoration of normal growth control in transformed tissues. One area that has been intensively studied in recent years is biological modifiers of cancer growth which are designed to retard proliferation [Novichenko et al., 1995], to induce differentiation of these cells to a quiescent, nondividing stage [Liu et al., 1994; Samid et al., 1993], and/or to promote cell death in malignant or premalignant cells [Welsh et al., 1994; Li et al., 1995]. One of these potential biological modifiers is the seco-steroid hormone 1α,25 dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) [Niles, 1995; Mangelsdorf et al., 1995] which is a key regulator of calcium homeostasis. It has also been found to have effects on the growth and differentiation of many normal and malignant tissues. This seco-steroid initiates genomic responses through a specific nuclear vitamin D_3 receptor (VDR) acting as a ligand-inducible transcription factor which in turn interacts with a vitamin D_3 response element (VDRE) contained within the promoter/enhancer region of target genes and thereby regulates specific gene transcription. These responses include the inhibition of proliferation and induction of differentiation of various tis-

Abbreviations: CDKI, cyclin-dependent kinase inhibitor; ED_{50}, estimated dose; FACS, fluorescence activated cell sorter; PI, propidium iodide; VDR, vitamin D receptor; VDRE, vitamin D response element; 1α,25$(OH)_2$, vitamin D_3.

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sue types, including cancerous and precancerous cells. For example, $1\alpha,25(OH)_2D_3$ can inhibit the growth and/or induce differentiation in vitro of cancer cells from the human hematopoietic system, breast, colon, skin, brain, and prostate [Norman et al., 1990; Jung et al., 1994; Brenner et al., 1995; James et al., 1994; Wali et al., 1995; Thomas et al., 1994; Shabahang et al., 1994; Yu et al., 1995; Naveilhan et al., 1994; Skowronski et al., 1995; Feldman et al., 1995].

Several genes have been identified that contain a VDRE within their promoter/enhancer region. An example of a specific, genomic effect of $1\alpha,25(OH)_2D_3$ includes cell cycle arrest in G_1. Many factors can lead to a cell cycle arrest, but the cyclin-dependent kinase inhibitors (CDKIs) known as $p21^{(waf1)}$ and $p27^{(kip1)}$ are pivotal to this process; the $p21^{(waf1)}$ gene contains a VDRE within its promoter region [Liu et al., 1996b]. We and others have demonstrated both transcriptional and translational mechanisms for increased expression of $p21^{(waf1)}$ and $p27^{(kip1)}$ in response to $1,25(OH)_2D_3$ in the HL-60 and U-937 myeloid leukemia cell lines and the LNCaP prostate cancer cell line [Hengst and Reed, 1996; Wang et al., 1996; Campbell et al., in press].

One major focus of research in the field of vitamin D_3 and cancer has been to identify analogs of $1,25(OH)_2D_3$ that have prominent antiproliferative effects against cancer cells without resulting in lethal hypercalcemia when administered in vivo at pharmacologically active doses. The overwhelming majority of analogs examined thus far have been derived by chemical synthesis. Within a cell, $1,25(OH)_2D_3$ readily undergoes metabolism along several different metabolic pathways, as described recently in our previous paper [Siu-Caldera et al., 1995]. All of these pathways were thought to be catabolic, and the intermediate metabolites were believed to have little or no biological activity.

Recently, we have isolated a major natural intermediate of a potent precursor analog ($1\alpha,25(OH)_2$-16-ene-D_3) formed through the C-24 oxidation metabolic pathway using a rat kidney perfusion biotransformation system [Caldera et al., 1996]. This intermediary metabolite, $1\alpha,25(OH)_2$-24-oxo-16-ene-D_3, is significantly resistant to further metabolism and therefore accumulates. This metabolite shares approximately the same level of potency as its parental analog ($1\alpha,25(OH)_2$-16-ene-D_3) in inhibiting proliferation and inducing differentiation of the human leukemic cell line RWLeu-4 and in transactivating a VDRE reporter construct [Caldera et al., 1996]. Most interestingly, the 24-oxo metabolite had reduced calcemic activity as compared to its parental analog [Lemire et al., 1994]. Therefore, the 24-oxo metabolites appear to allow a separation of the antiproliferative genomic effects of vitamin D_3 compounds from their hypercalcemic side effects.

We have now undertaken a more comprehensive study of the activities of these 24-oxo metabolites compared to their precursor analogs. The $1\alpha,25(OH)_2D_3$ and two potent analogs (containing either a 16-ene or a 20-*epi* modification to the side chain) were compared to their corresponding 24-oxo metabolites for activity against cell lines of three types of cancers: human myeloid leukemia (HL-60), breast cancer (MCF-7 and MDM-MB-436), and prostate cancer (LNCaP and DU-145).

Initially, we compared the abilities of the vitamin D_3 analogs and their corresponding 24-oxo metabolite to inhibit clonal proliferation of the cancer cells. Furthermore, their effects on the cell cycle, differentiation, and apoptosis of the cancer cells were studied. We particularly focused on one of the breast cancer cell lines (MCF-7), examining modulation of its $p21^{(waf1)}$ and $p27^{(kip1)}$ and the expression of BAX, an apoptosis-inducing protein.

Two other hormonally regulated proteins possibly important in the growth regulation of normal tissue were studied: the cell-surface adhesion molecule E-cadherin and the recently identified breast cancer susceptibility protein, BRCA1 [Miki et al., 1994]. These putative tumor suppressors are commonly mutated in several types of tumors; for example, the BRCA1 gene is frequently mutated in breast and ovarian cancers [Marquis et al., 1995]. These cancers are also often associated with altered expression of E-cadherin. For example, 50% of metastatic breast cancers have downregulated expression of E-cadherin [Berx et al., 1995]. Deregulated expression of both proteins is associated with a more transformed, less differentiated phenotype [Sitonen et al., 1996; Eisinger et al., 1996].

MATERIALS AND METHODS

Cells

All cell lines were obtained from ATCC (Rockville, MD) and maintained according to their

recommendations. HL-60 cells are leukemic promyelocytes isolated from a patient with acute myeloid leukemia. MCF-7 and MDA-MB-436 were developed from pleural effusions of patients with metastatic adenocarcinoma of the breast. LNCaP has been derived from a lymph node metastasis from a patient with hormonally refractory prostate cancer. DU-145 was established from a prostate cancer metastatic to the brain. Salient features of these cell lines are summarized in Table I.

Vitamin D_3 Analogs and Their 24-Oxo Metabolites

The concentration of all compounds was determined via absorbance spectroscopy at 265 nm. 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-16-ene-D_3 were synthesized at Hoffmann LaRoche, (Nutley, NJ), and 1α,25-$(OH)_2$-20-*epi*-D_3 was synthesized at Leo Pharmaceuticals (Ballerup, Denmark). The 24-oxo metabolites of 1α,25-$(OH)_2$-D_3 and its two analogs were produced using the kidney perfusion system, as previously described [Caldera et al., 1996]. All the compounds were then kept in stock vials at 10^{-3} M in ethanol at −20°C in the dark. For experimental use, they were diluted in media with 10% fetal calf serum. The compounds were assigned number codes as follows: cpd. 1, 1α,25-$(OH)_2$-D_3; cpd. 1a, 1α,25-$(OH)_2$-24-oxo-D_3; cpd. 2, 1α,25-$(OH)_2$-16-ene-D_3; cpd. 2a, 1α,25-$(OH)_2$-24-oxo-16-ene-D_3; cpd. 3, 1α,25-$(OH)_2$-20-*epi*-D_3; cpd. 3a, 1α,25-$(OH)_2$-24-oxo-20-*epi*-D_3.

Effect of Vitamin D_3 Analogs and Their 24-Oxo Metabolites on Clonal Growth of Cancer Cells in Soft Agar

Potency of vitamin D_3 analogs and their 24-oxo metabolites was determined by extensive dose-response studies in soft agar. Target cancer cells from 80% confluent cultures were plated into 24-well, flat-bottom plates using a two-layer soft agar system with a total volume of 400 µl, as described previously [Munker et al., 1986]. The cells were maintained in their respective media. The feeder layer was prepared with agar (1%) that had been equilibrated to 42°C. Prior to addition of this layer to the plate, the vitamin D_3 analogs and metabolites were pipetted into the wells. After 10 days (HL-60) and 14 days (breast and prostate) of growth in soft agar, the colonies (≥50 cells) were counted with an inverted microscope. All experiments were done at least three times in triplicate dishes per experimental point.

Mechanism of Inhibition

Effect of vitamin D_3 analogs on cell cycle and cell cycle–related proteins. The vitamin D_3–sensitive cell lines HL-60, MCF-7, and LNCaP were exposed to either analog or metabolite, and the cell cycle distribution was determined. DNA was stained with propidium iodide (PI) after a total of 5×10^5 subconfluent, exponentially proliferating cells were either cultured with the vitamin D_3 compounds (10^{-7} M for 3 days) or left untreated (control). Total cells, both in the media and remaining adherent, were harvested, washed, resuspended in PBS, and stained with trypan blue, and viable and nonviable cells were counted. The cells were adjusted to a final concentration of 1×10^6 viable cells/ml and fixed in a 2:1 (vol/vol) ratio in chilled methanol overnight prior to staining with PI in the presence of RNAse One (Promega, Madison, WI). Cell cycle distribution was determined on a Becton-Dickinson (Braintree,

TABLE I. Summary of Key Features of Target Cancer Cell Lines*

Tissue type	Leukemia	Prostate cancer		Breast cancer	
	HL-60	LNCaP	DU-145	MCF-7	MDA-MB-436
Expression VDR	wt	wt	wt	wt	wt
Sensitive to clonal inhibition by 1α,25-$(OH)_2$-D_3	[a]	[b]	[c]	[a]	[c]
Expression p53	ko	wt	ko	wt	NI
Expression Rb	wt	wt	ko	wt	NI
Sensitive to sex hormone	NI	[d]	[e]	[d]	[e]

*ko, functional knockout of gene; NI, not investigated; wt, wild-type expression.
[a]Extreme sensitivity to 1α,25-$(OH)_2$-D_3.
[b]Moderate sensitivity to 1α,25-$(OH)_2$-D_3.
[c]Insensitive to 1α,25-$(OH)_2$-D_3.
[d]Sensitive to androgen (LNCaP) or estrogen (MCF-7).
[e]Insensitive to androgen (LNCaP) or estrogen (MCF-7).

MA) FACScan Flow Cytometer and CellFIT Cell-Cycle Analysis software.

Expression of $p21^{(waf1)}$ in HL-60 and MCF-7 was examined after 48 h exposure to the precursor analog or metabolites (10^{-7} M). In a similar manner, $p27^{(kip1)}$ was examined in MCF-7 after 0–4 days exposure to the same vitamin D_3 compounds. Control and test cultures of both HL-60 and MCF-7 cells were maintained for the same durations at parallel cell numbers. Cellular lysates from cultures were subjected to SDS-PAGE. Briefly, extracts from 1.5×10^6 cells were boiled in sample buffer for 5 min and loaded onto a 12.5% SDS-polyacrylamide gel. After electrophoresis at 150 V, the proteins were transferred to Immobion-P (Millipore, Bedford, MA) membrane, blocked with Tris-buffered saline containing Tween 20 (0.1%) and gelatin (1%) at pH 7.5 for 1 h, and then incubated with antibodies to either $p21^{(waf1)}$ (Oncogene Research Products) or $p27^{(kip1)}$ (Santa Cruz Biotechnology, Santa Cruz, CA) [Upadhyay et al., 1995; Fang et al., 1996]. The proteins were detected using an ECL chemiluminescence system (Amersham Life Sciences, Arlington Heights, IL). To ensure even loading of proteins, nonspecific bands were compared, and the membrane was stained afterwards with Ponceau S. Densitometry was performed on bands to quantify the changes in detected protein.

Effect of vitamin D_3 analogs on differentiation. Induction of differentiation was measured by the modulation of surface expression of CD 11b on HL-60 cells, secretion of prostate specific antigen (PSA) by LNCaP cells, and modulation of E-cadherin and BRCA1 in MCF-7 cells. Expression of the surface marker CD 11b was measured by direct immunofluorescence. Approximately 5×10^5 cells were incubated with a saturating concentration of murine CD 11b antibody (Carpenteria, CA) for 45 min on ice, followed by incubation with a goat anti-mouse FITC-conjugated secondary antibody. A FITC-conjugated isotype control was used. Immunofluorescence was analyzed with a Becton-Dickinson FACScan Flow Cytometer using LYSIS II software.

Changes in PSA were measured by plating LNCaP cells (1×10^5) into six-well dishes in 3 ml of media with either cpd. 2 (1α,25-$(OH)_2$-16-ene-D_3), cpd. 2a (1α,25-$(OH)_2$-24-oxo-16-ene-D_3) (10^{-7} M), or media alone (control). Media were harvested at day 4, and PSA levels were measured by a TANDEM-E ELISA method; viable cells were counted to normalize PSA levels.

Modulation of expression of the BRCA1 and E-cadherin proteins was measured by Western blot analysis as indicated above, using antibodies for E-cadherin (Transduction Laboratories, Lexington, CA) and BRCA1 (Santa Cruz Biotechnology) [Tamm et al., 1994; Chen et al., 1995].

Effect of vitamin D_3 analogs on apoptosis. HL-60, MCF-7, and LNCaP cells were exposed to the vitamin D_3 analog and its 24-oxo metabolite (10^{-7} M). Fresh media and vitamin D_3 compounds were added on day 2 of culture, and at that time any detached cells were placed back in the test cultures. On day 4, DNA fragmentation was measured as described previously [Li and Daryzynkiewicz, 1995]. Briefly, total cells, both in the media and those remaining adherent to the plastic dishes, were harvested and fixed in 1% methanol-free formaldehyde for 15 min and washed in phosphate buffered saline (PBS). The cell concentration was corrected to 1×10^6 cells/ml, and these cells were fixed in 5 ml of 70% ethanol. Single- and double-strand DNA breaks were labeled with bromodeoxyuridine triphosphate (BrD-UTP) for 40 min at 37°C with terminal transferase (Boehringer-Mannheim, Indianapolis, IN). The cells were permeabilized with a 0.3% solution of Triton-X 100 in 0.5% bovine serum albumin (BSA)/PBS. Cells that had breaks in DNA were tagged by the incorporation of BrDU and were identified with a FITC-conjugated anti-BrDU antibody. Cells were stained with PI for 30 min, and green fluorescence was measured by FACS analysis at 510–550 nm. As a positive control, cells were treated with etoposide (50 μg/ml for 2 days).

Modulation of expression of the apoptosis-associated protein BAX was measured by Western blot analysis as indicated above using antibody for BAX (Santa Cruz Biotechnology) [Elstner et al., 1996].

RESULTS

Clonal Inhibition of Proliferation of Leukemic, Breast, and Prostate Cancer Cells Mediated by Vitamin D_3 Analogs and Their 24-Oxo Metabolites

Three pairs of vitamin D_3 compounds and their corresponding 24-oxo metabolites were examined for their effects on the clonal proliferation of a leukemic, two breast, and two prostate

TABLE III. Cell Cycle Distribution by Mediated Vitamin D_3 Analogs and Their 24-Oxo Metabolites*

Vitamin D_3 compounds	HL-60 (%)		MCF-7 (%)		LNCaP (%)	
Control	G_0/G_1	49 ± 0.1	G_0/G_1	38 ± 1.5	G_0/G_1	66 ± 1.5
	S	37 ± 0.3	S	35 ± 0.8	S	27 ± 0.7
	G_2/M	14 ± 0.3	G_2/M	26 ± 0.9	G_2/M	10 ± 1.2
2. 1α,25-$(OH)_2$-16-ene-D_3					G_1/G_1	77 ± 0.7
					S	18 ± 0.9
					G_2/M	5 ± 1.1
2a. 1α,25-$(OH)_2$-24-oxo-16-ene-D_3					G_0/G_1	78 ± 1.0
					S	13 ± 1.1
					G_2/M	8 ± 0.6
3. 1α,25-$(OH)_2$-20-*epi*-D_3	G_0/G_1	67 ± 2.1	G_0/G_1	52 ± 1.6		
	S	26 ± 0.4	S	26 ± 1.4		
	G_2/M	6 ± 1.3	G_2/M	21 ± 1.3		
3a. 1α,25-$(OH)_2$-24-oxo-20-*epi*-D_3	G_0/G_1	65 ± 0.2	G_0/G_1	51 ± 0.4		
	S	26 ± 0.5	S	27 ± 1.0		
	G_2/M	9 ± 0.4	G_2/M	22 ± 0.7		

*Cells (HL-60, leukemia cells; MCF-7 and LNCaP breast and prostate cancer cells) were plated in triplicate wells and grown in the presence or absence (control) of the test compounds. After 3 days, the cell cycle distribution was measured (Materials and Methods).

increase in CD 11b expression with both compounds (data not shown). Prostate-specific antigen (PSA) is a secreted protease of the normal prostate which has also been used as a prostate-specific tumor marker. Investigators have suggested that it may also be a differentiation marker of normal prostate development [Wang et al., 1996]. Similarly, LNCaP was cultured with either cpd. 2 or 2a (10^{-7} M, 4 days) and produced an equal increase in PSA with each compound (approximately 70%) (data not shown). Thus, both vitamin D_3 analogs and their 24-oxo metabolites were equally able to induce differentiation of HL-60 and LNCaP cells.

E-cadherin is a cell surface adhesion molecule that is essential for maintaining the cell-cell adhesion system, and its decreased expression has been associated with metastatic disease [Sitonen et al., 1996]. The breast cancer cell line MCF-7 showed an approximately 180% increase in the expression of E-cadherin after 4 days exposure (10^{-7} M) to either cpd. 3 or 3a (Fig. 3).

The BRCA1 protein has a putative tumor suppressor role in breast and ovarian cancer cells, although the exact function of this protein is unclear [Jensen et al., 1996]. One of the most interesting findings of the present study is the inverse modulation of BRCA1 protein in response to either cpd. 3 or 3a (Fig. 4). The parent analog, cpd. 3 (1α,25-$(OH)_2$-20-*epi*-D_3) (10^{-7} M), induced a modest increase of BRCA1 expression (130% at 48 h), and this was sustained at 96 h of culture. However, the corresponding 24-oxo metabolite, cpd. 3a (1α,25-$(OH)_2$-24-oxo-20-*epi*-D_3) (10^{-7} M), demonstrated the opposite behavior, dramatically decreasing BRCA1 levels by 25% at 48 h and 75% by 96 h of culture.

Effect of vitamin D_3 analogs and 24-oxo metabolites on apoptosis. Cells arrested in their progression through the cell cycle may undergo one of several fates, including apoptosis, which was investigated in a similar manner to the cell cycle analysis. Three target cell lines (MCF-7, HL-60, and LNCaP) were exposed to the same vitamin D_3 analogs or their 24-oxo metabolites used for the cell cycle analysis (10^{-7} M, 4 days). Only MCF-7 cells, not HL-60 or LNCaP cells, underwent apoptosis in response to either the analog or metabolite as detected by FACS analysis of DNA single- and double-strand breaks (Fig. 5). The level of apoptosis in MCF-7 cells was approximately 9.5 ± 0.3% with cpd. 3, but its 24-oxo metabolite (cpd. 3a) significantly increased (doubled) ($P \leq 0.02$) the proportion of apoptotic cells to 23 ± 1.6%. Enhanced BAX expression has been associated with apoptosis. Correlated with the induction of apoptosis of MCF-7, exposure of these cells to cpd. 3 increased by 190% (48 h) and 150% (96 h) the expression of BAX as determined by Western blot. The 24-oxo metabolite (cpd. 3a) increased by 150% (96 h) the levels of BAX (Fig. 6).

DISCUSSION

In this study, we have compared the biological effects of 1α,25-$(OH)_2$-D_3 and two of its syn-

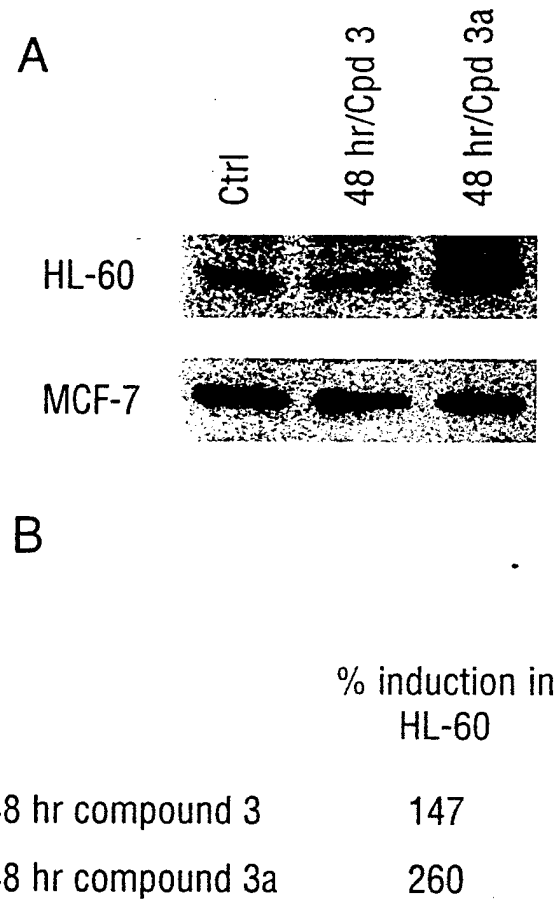

Fig. 2. Modulation of $p21^{(waf)}$ protein expression in HL-60 and MCF-7 cells. HL-60 and MCF-7 cells were treated with either cpd. 3 (1α,25(OH)$_2$-20-*epi*-D$_3$) or cpd. 3a (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$) at 10^{-7} M for the indicated time periods or left untreated. **A:** Cell lysates were resolved by SDS-PAGE, and $p21^{(waf)}$ was detected by Western blot analysis using the antibody described in Materials and Methods. **B:** Densitometry was performed on bands to quantify the changes in detected protein.

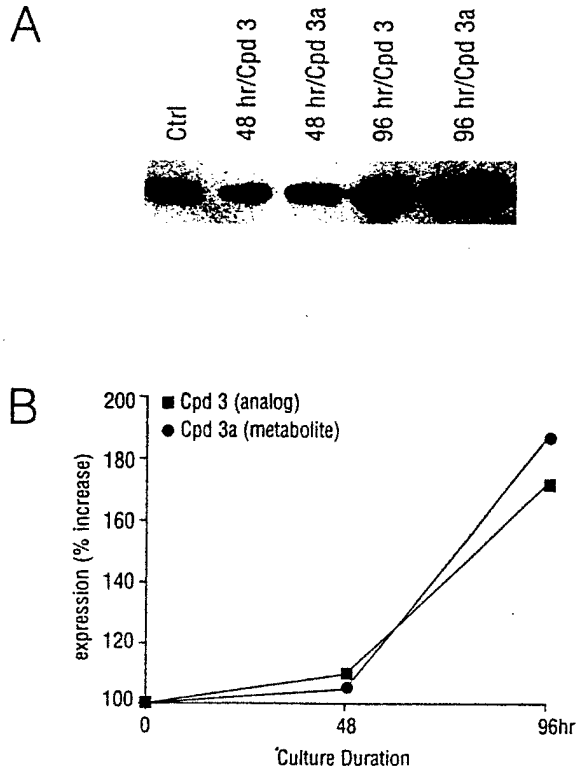

Fig. 3. Differentiation of MCF-7 cells as measured by modulation of E-cadherin protein expression. E-cadherin expression by MCF-7 cells was measured after treatment with either cpd. 3 (1α,25(OH)$_2$-20-*epi*-D$_3$) or cpd. 3a (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$) at 10^{-7} M for the indicated durations or left untreated. **A:** Cell lysates were resolved by SDS-PAGE, and E-cadherin was detected by Western blot analysis using the antibody described in Materials and Methods. **B:** Densitometry was performed on bands to quantify the changes in detected protein.

thetic analogs and their corresponding 24-oxo metabolites on leukemic, breast, and prostate cancer cell lines. The initial analysis of activity utilized the exquisitely sensitive soft agar clonogenic assay which examines the clonal capacity of single cells to undergo at least six divisions. The 24-oxo metabolites generally had the same activity as their parental analogs. However, several exceptions are very interesting. The DU-145 cell line is resistant to growth inhibition by most vitamin D$_3$ analogs [Campbell et al., in press]. In contrast, the 24-oxo metabolite of 1α,25-(OH)$_2$-D$_3$ (cpd. 1a [1α,25(OH)$_2$-24-oxo-D$_3$]) but not 1α,25(OH)$_2$-D$_3$ markedly inhibited clonal growth of these cells (ED$_{50}$, 50 nM). Similarly, the breast cancer cell line MDA-MB-436 is resistant to growth inhibition by most vitamin D$_3$ analogs [Elstner et al., 1995]. However, cpd. 3a (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$) but not cpd. 3 [1α,25(OH)$_2$-20-*epi*-D$_3$] inhibited the clonal growth of these cells (ED$_{50}$, 5 nM). The ability of these two 24-oxo metabolites to inhibit the clonal growth of other cancer cell lines known to be resistant to most vitamin D$_3$ compounds is now being explored.

Cell cycle analysis of the three vitamin D$_3$–sensitive cell lines revealed that the precursor vitamin D$_3$ analogs and their 24-oxo metabolites had similar potencies to induce a G1 arrest of cancer cells. Likewise, they had similar abilities to induce differentiation, as measured by an increased expression of CD 11b on HL60 cells and increased secretion of PSA by LNCaP cells.

Apoptosis did not significantly increase in either HL-60 or LNCaP cells cultured with either the vitamin D$_3$ analogs or their 24-oxo metabolites. However, the breast cancer cell

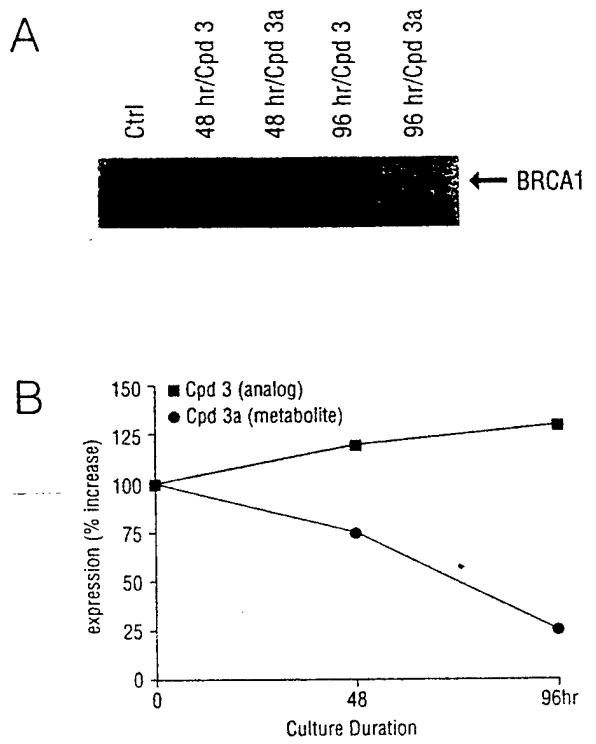

Fig. 4. Modulation of BRCA1 protein expression in MCF-7 cells. BRCA1 expression by MCF-7 cells was measured after treatment with either cpd. 3 (1α,25(OH)$_2$-20-*epi*-D$_3$) or cpd. 3a (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$) at 10^{-7} M for the indicated durations or left untreated. **A:** Cell lysates were resolved by SDS-PAGE, and BRCA1 was detected by Western blot analysis using the antibody described in Materials and Methods. **B:** Densitometry was performed on bands to quantify the changes in detected protein.

line MCF-7 doubled its level of apoptosis (23 ± 1.6%) when cultured with the 24-oxo metabolite of cpd. 3 (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$). Taken together, the data on clonal inhibition, cell cycle arrest, differentiation, and apoptosis indicate that the 24-oxo metabolites are at least as potent as their precursor vitamin D$_3$ compounds. Therefore, this study clearly shows that metabolites of 1α,25-(OH)$_2$-D$_3$ can be active, and these findings contradict for the first time the previous conventional wisdom. Furthermore, the data also suggest that the 24-oxo metabolites may possess unique antiproliferative characteristics. The full spectrum of these features is now being explored.

To examine further the molecular effects of the compounds, we examined the effect of cpd. 3 and cpd. 3a on levels of expression of several CDKIs which are integrally associated with cell cycle arrest, apoptosis, and differentiation. Expression of p21$^{(waf1)}$ has been reported to be upregulated by 1α,25(OH)$_2$-D$_3$ in HL-60 cells [Munker et al., 1996]. We confirmed this finding using cpd 3 (1α,25(OH)$_2$-24-oxo-D$_3$) and also found that the 24-oxo metabolite could also increase the levels of expression of p21$^{(waf1)}$ in HL-60 cells. By contrast, the MCF-7 breast cancer cells did not increase their expression of p21$^{(waf1)}$ after culture with either the vitamin D$_3$ analog or its 24-oxo metabolite. Previous studies have suggested that induction of expression of p21$^{(waf1)}$ can have an apoptosis-inhibitory role during the process of differentiation [Wang et al., 1996]. Perhaps the apoptosis is induced by vitamin D$_3$ compounds in MCF-7 cells because p21$^{(waf1)}$ is not modulated in these cells. Likewise, expression of p27$^{(kipl)}$ was not increased by the vitamin D$_3$ compounds in these cells, although we [Siu-Caldera et al., 1995] and others [Hengst and Reed, 1996; Campbell et al., in press] have found enhanced expression of this CDKI in other cells types cultured with vitamin D$_3$ compounds. Another key regulatory protein in the process of apoptosis is BAX. It was slightly upregulated after treatment with either 1α,25(OH)$_2$-20-*epi*-D$_3$ or 1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$. Taken together, we still do not have a clear understanding of the molecular events responsible for the growth inhibition of cancer cells mediated by the vitamin D$_3$ analogs and their 24-oxo metabolites. Most of our target cancer cells are inhibited in their clonal growth at the G$_1$ stage of the cell cycle by the vitamin D$_3$ compounds.

The effect of the vitamin D$_3$ analogs and their 24-oxo metabolites on expression of E-cadherin and BRCA1 was also examined, as both have importance in the development or progression of breast cancer. Murine gene-knockout studies have shown both proteins to be essential for embryonic development [Liu et al., 1996a]. E-cadherin controls growth by binding with the cytoplasmic integrin proteins. This interaction can modulate cellular proliferation by reacting internally with other proteins such as the APC tumor suppressor gene [Su et al., 1993]. Both the analog and its 24-oxo metabolite induced an equally upregulated expression of E-cadherin, which may contribute to their comparable level of cell cycle arrest of the MCF-7 cells.

The exact function of the BRCA1 protein is less well characterized. Retroviral studies have demonstrated that wild-type but not mutant forms of the BRCA1 gene inhibit growth in vitro of some breast and ovarian cancer cell lines [Holt et al., 1996]. To our knowledge, we

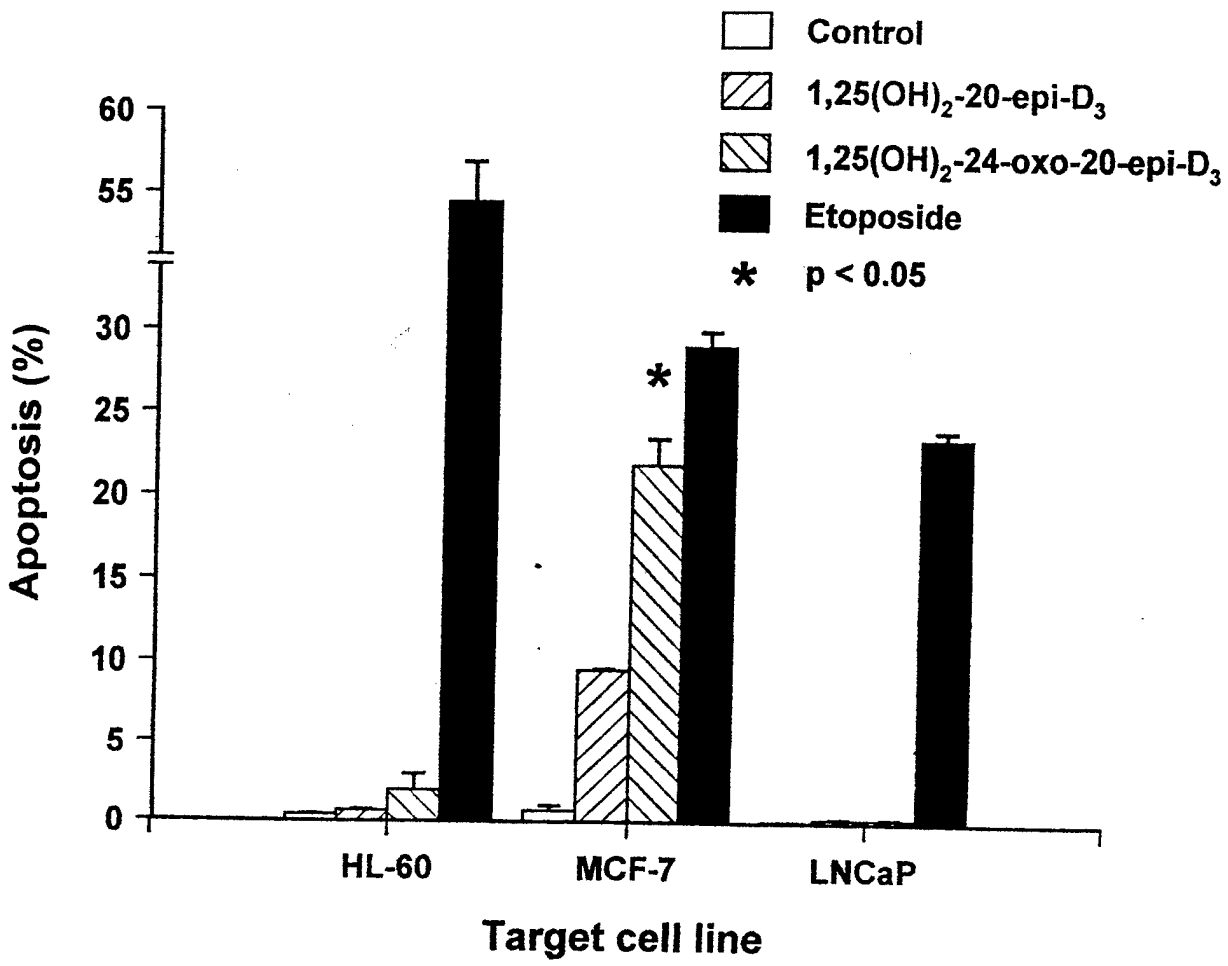

Fig. 5. Apoptosis measurements. HL-60, MCF-7, and LNCaP were exposed to cpd. 3 (1α,25(OH)$_2$-20-*epi*-D$_3$) or cpd. 3a (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$) (HL-60 and MCF-7) and cpd. 2 (1α,25-(OH)$_2$-16-ene-D$_3$) and cpd. 2a (1α,25-(OH)$_2$-24-oxo-16-ene-D$_3$) (LNCaP) (10^{-7} M, 4 days), and apoptosis was measured as described in Materials and Methods. Each cell line was treated with 50 μg/ml etoposide as a positive control and also evaluated for apoptosis.

show for the first time that vitamin D$_3$ compounds can modulate expression of BRCA1. The modulation of this protein revealed a striking difference when comparing the vitamin D$_3$ analog and its 24-oxo metabolite; the 20-epi analog slightly increased levels of BRCA1, whereas the 24-oxo metabolite dramatically downregulated expression of BRCA1. The mechanism by which this disparate activity occurred requires further analysis. Negative regulation by vitamin D$_3$ has been demonstrated by us to be the result of posttranscriptional control of protein expression of [Tobler et al., 1988]. Others have shown it to be the result of direct transcriptional control [Peleg et al., 1993; Liu et al., 1996c]. Thus, it is not without precedent that a vitamin D$_3$ compound might have a negative regulatory role on protein expression. Perhaps the analog and its 24-oxo metabolite induce a novel conformation of the VDR, thereby allowing their interaction with different sets of VDRE. Recently, estrogen (another steroid hormone) and one of its metabolites have been shown to interact with different response elements. This allows the parental hormone and the metabolite to exert differing cellular and tissue-specific responses [Yang et al., 1996]. Potentially, vitamin D$_3$ or its analogs upregulate expression of BRCA1; then, by conversion to the 24-oxo metabolite, the cell may negatively regulate the same gene, possibly by upregulation of other BRCA1 negative-regulatory proteins. We are currently investigating this finding more fully.

In summary, we have compared for the first time three vitamin D$_3$ compounds and their 24-oxo metabolites and defined their biological potency against cancer cells. In general, the

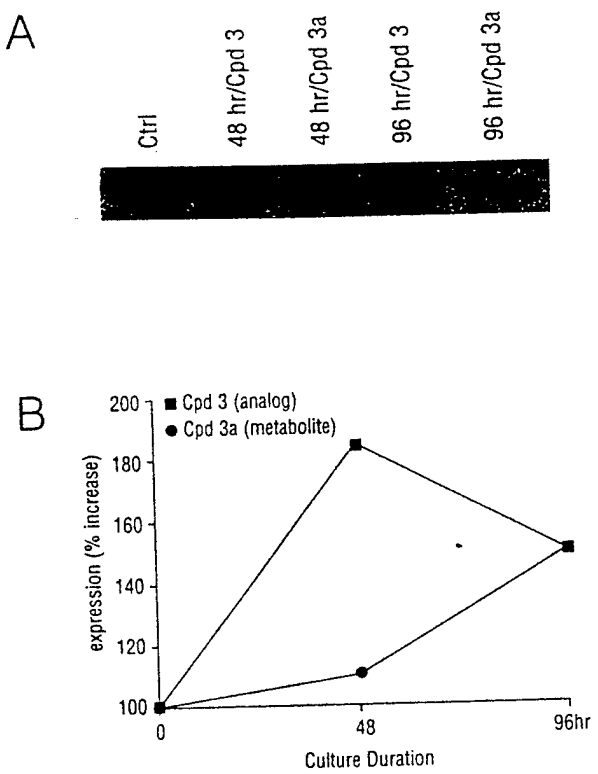

Fig. 6. Expression of BAX. BAX expression by MCF-7 cells was measured after treatment with either cpd. 3 (1α,25(OH)$_2$-20-*epi*-D$_3$) or cpd. 3a (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$) at 10^{-7} M for the indicated durations or left untreated. **A:** Cell lysates were resolved by SDS-PAGE, and BAX was detected by Western blot analysis using the antibody described in Materials and Methods. **B:** Densitometry was performed on bands to quantify the changes in detected protein.

24-oxo metabolites, which had previously been thought to be biologically inactive, were shown to be at least as potent as their parental vitamin D_3 compounds as measured by their inhibition of cancer cell clonal proliferation, arrest of the cell cycle, and induction of differentiation and apoptosis. At the molecular level, the precursor compounds and their 24-oxo metabolites can behave in a disparate manner, as exemplified by the modulation in BRCA1 expression in breast cancer cells. Previously, we have shown that cpd. 2a (1α,25-(OH)$_2$-24-oxo-16-ene-D$_3$) [Lemire et al., 1994] and cpd. 3a (1α,25-(OH)$_2$-24-oxo-20-*epi*-D$_3$) (manuscript in preparation) have lower in vivo calcemic effects than their precursor analogs; thus, these metabolites are very attractive candidates for future therapeutic investigation.

ACKNOWLEDGMENTS

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Combination Therapy of a Vitamin D_3 Analog and All-Trans-Retinoic Acid: Effect on Human Breast Cancer in Nude Mice*

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Abstract. *Background: Vitamin D_3 analogs and all-trans-retinoic acid (ATRA) are able to inhibit the growth of a variety of malignant cells. Material and Methods: We examined the ability of three vitamin D_3 analogs to inhibit the growth of a human mammary cancer cell line (MCF-7) in Beige Nude xid (BNX) mice either alone or with ATRA. Vitamin D_3 analogs 1,25 dihydroxyvitamin D_3 (code name, compound C), 1,25(OH)$_2$-16-ene-23-yne-19-nor-26,27-F_6-D_3 (compound LH) and 24a,26a,27a,-trihomo-22,24-diene-1,25(OH)$_2$$D_3$ (EB1089) were used. Results: The antitumor effect of ATRA alone was greater than that of either of the vitamin D_3 analogs alone, and an additive effect was observed when a vitamin D_3 analog and ATRA were administered together. EB1089 was the most potent vitamin D_3 analog; and EB1089 plus ATRA was the most potent combination decreasing the tumor mass nearly 3-fold compared to tumors of diluent control mice. None of the animals became hypercalcemic. Their complete blood counts, serum electrolyte analysis as well as their liver and renal functions were all fairly similar and within the normal range. Conclusion: This combination of a vitamin D_3 analog and ATRA has the potential to be an adjuvant therapy for breast cancer.*

Breast cancer is one of the most common malignant diseases of women in the United States, and it is associated with an appreciable morbidity and mortality. Chemotherapy and endocrine therapy play a central role in the treatment of breast cancer either in the adjuvant setting or in those who failed endocrine manipulation. The active seco-steroid hormone vitamin D_3 metabolite, 1,25 dihydroxyvitamin D_3 [1,25(OH)$_2$$D_3$] (code name, compound C), is a well- known regulator of calcium homeostasis and bone metabolism (1). 1,25(OH)$_2$$D_3$ is also involved in the regulation of proliferation and differentiation of a large variety of cells and tissues (2). Most breast cancer cell lines and more than 80% of breast tumors express high affinity intracellular vitamin D receptors (VDR) (3-6). One study suggested that patients whose breast cancer cells expressed VDR had significantly longer disease-free survival than those with receptor-negative tumors (7). Preliminary data suggest that a VDR polymorphism is associated with the development of breast cancer.

Vitamin D_3 analogs decreased the development and progression of breast cancer and other carcinomas *in vivo* (8-9), and inhibited the metastatic spread of tumor cells (7). However, the hypercalcemic effect of 1,25(OH)$_2$$D_3$ has prevented its application as a pharmaceutical agent. Recently, vitamin D_3 analogs have been developed that can inhibit cellular proliferation and induce differentiation without causing hypercalcemia (8,10,11). The analog EB1089 [24a,26a,27a,-trihomo-22,24-diene-1,25(OH)$_2$$D_3$] has a wide anticancer spectrum *in vitro* (12-15). The analog is about 7- to 50-fold more potent than the parental 1,25(OH)$_2$$D_3$ *in vitro* (12); furthermore, it is undergoing Phase I and II anticancer trials. It has similar effects on serum calcium levels compared to 1,25(OH)$_2$$D_3$ (12).

Analog LH [1,25(OH)$_2$-16-ene-23-yne-19-nor-26,27-F_6-D_3] appears to have an expanded range of anticancer activity compared to most other vitamin D_3 compounds (12,16,17). This may result from the six fluorines added to the end of the side chain. This also increases its hypercalcemic activity, thus requiring a balance between toxicity versus anticancer activity.

The vitamin A-derived retinoids play an important role in regulating a broad range of biological processes, including cell growth and differentiation in a variety of cancer cells (18). Retinoids inhibit the growth and invasion of cancer cells (19,20) and are clinically useful for the treatment and prevention of several human malignancies including acute promyelocytic leukemia, recurrent head and neck cancer and

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Abbreviations used: 1,25(OH)$_2$$D_3$, 1,25 dihydroxyvitamin D_3; VDR, vitamin D receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor.

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Key Words: Vitamin D_3 analogs, all-trans-retinoic acid, breast cancer, BNX mouse.

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leukoplakia (21-23). *In vitro*, retinoids inhibit breast cancer cell growth (24,25) and induce apoptosis (26). Retinoids exert their effects by interacting with specific retinoic acid receptors (RARs) and retinoic acid X receptors (RXRs) (27). These receptors are ligand-dependent, DNA-binding proteins which regulate the expression of target genes by binding to retinoic acid responsive elements (RAREs) in the promoters of these target genes. ATRA binds and activates RARs, whereas 9-*cis*-retinoic acid binds and activates both RARs and RXRs. The RAR and RXR isotypes are expressed in breast cancer cells. In general, RAR RNA expression is higher in estrogen receptor (ER)-positive cells than ER-negative cells (28). RXRs RNAs have been detected in both ER positive and ER negative breast cancer cells (29).

Others as well as ourselves have shown that the combination of a vitamin D_3 analog and a retinoid can have an additive or synergistic inhibitory effect on a variety of cancer types including breast cancer (30,31). We studied the ability of the combination of a vitamin D_3 analog and ATRA to inhibit the growth of human breast cancer *in vivo*. We believe that this is the first report showing that one of several active vitamin D_3 analogs combined with ATRA is efficacious in inhibiting the growth of human MCF-7 breast tumors in BNX nude mice.

Materials and Methods

Chemicals. The $1,25(OH)_2D_3$ (compound C) and $1,25(OH)_2$-16-ene-23-yne-19-nor-26,27-F6-D_3 (compound LH) were synthesized by Milan Uskokovic at Hoffmann- La Roche Inc. (Nutley, NJ) and 24a,26a,27a,-trihomo-22,24-diene-$1,25(OH)_2D_3$ (EB1089) was made by LEO Pharmaceutical Products (Ballerup,Denmark). Compounds C and LH were dissolved in absolute ethanol at 10^{-3} M as a stock solution, which was stored at-20°C and protected from light. EB1089 was dissolved in propylene glycol: 0.05M Na_2HPO_4 (80:20) at 0.1 mg/ml as a stock solution, which was stored at below 5°C and protected from light. ATRA (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at 50 mg/ml and stored at -80°C and protected from light.

Cell line. MCF-7 human breast cancer cells were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) containing 10% bovine fetal serum, according to their recommendations in culture flasks with vented filter caps (Costar, Cambridge, MA).

Mice. Forty female BNX nu/nu mice at 8 weeks of age were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN), and maintained in pathogen-free conditions with irradiated chow.

Treatment protocol. Animals were bilaterally, subcutaneously injected with 5×10^6 MCF-7 cells/tumor in 0.1 ml Matrigel (Collaborative Biomedical Products, Bedford, MA). Before injection of cells, the animals received 300 rads whole body irradiation. Mice were divided randomly into 8 groups of 5 mice each, Groups included: diluent treated (control); ATRA; compound C [$1,25(OH)_2D_3$]; compound LH; EB1089; compound C + ATRA; compound LH + ATRA; and Group EB1089 + ATRA.

Vitamin D_3 analogs and ATRA were administered intraperitoneally every other day at the following doses: compound C, 0.05 μg/mouse; compound LH, 0.0125 μg/mouse; EB1089, 0.05 μg/mouse; and ATRA, 7.5 mg/mouse. The doses were chosen after a series of initial experiments determined the highest doses of the vitamin D_3 compounds and ATRA that could be given without causing either hypercalcemia or other major side-effects. One day after tumor injections, mice were treated with either one of the vitamin D_3 analogs alone, ATRA alone, or the combination of a vitamin D_3 analog and ATRA. Tumors were measured every week with vernier calipers. Tumor size was calculated by the formula: a x b x c, where a is the length and b is the width and c is the height in millimeters.

At the end of the experiment, blood was collected from the orbital sinus for chemistry and blood analyses, and animals were killed by CO_2 asphyxiation and tumor weights were measured after careful resection. Chemistry and blood analyses were measured by Dupont Analyst Benchtop Chemistry System, Dade International, Newark, DE and by Serono-Baker 9000 Diff, Biochem Immuno- Systems, Allen town, PA, respectively.

Histology. Tumors and normal organs from sacrificed mice were fixed in 10% neutral buffered formalin and embedded in paraffinwax prior to histologic sectining. Sections were stained with Hematoxylin and Eosin, and percentage of tumors showing necrosis or fibrosis was evaluated. Normal organs were evaluated for evidence of toxic damage including calcification.

Statistical analysis. The statistical significance of the differences were analyzed by the non- parametric Mann-Whitney U test.

Results

Figure 1 shows the effect of vitamin D_3 analogs and ATRA, either alone or in combination, on the size of MCF-7 tumors during nine weeks of therapy. At completion of the trial, all the treatment groups had statistically significantly smaller tumors than the control cohort. The most potent single agent was ATRA (Group B). The second in potency was EB1089 (Group E). An enhanced effect was observed when ATRA and vitamin D_3 analogs were administered together. In each case, the combination of ATRA and a vitamin D_3 analog suppressed tumor growth greater than either alone. All the treated groups were statistically different from the control group ($p<0.02$).

The results were similar when the effect of vitamin D_3 analogs and ATRA were evaluated by tumor weight at the conclusion of the study (Figure 2). Again, ATRA was the most potent single agent, and the combination of a vitamin D_3 analog and ATRA was more potent than either alone. Tumor weights in the combined treatment groups were approximately 35% to 40% of those in the control cohort and were statistically different from the control cohort ($p<0.002$). In addition, all the combined treatment groups were statistically different from all other groups ($p<0.005$).

Controls revealed poorly differentiated adenocarcinomas with small foci of necrosis and fibrosis which constituted less than 15% of the area of the sections (Figure 3A). Sections from mice treated with ATRA, compound C, and compound LH revealed necrosis and fibrosis involving approximately 30% of the tumor area, and numerous apoptotic bodies were present (Figure 3B, 3C). Tumors from mice treated with EB1089 were similar but in addition showed calcification, as did tumors from mice treated with compound C and ATRA

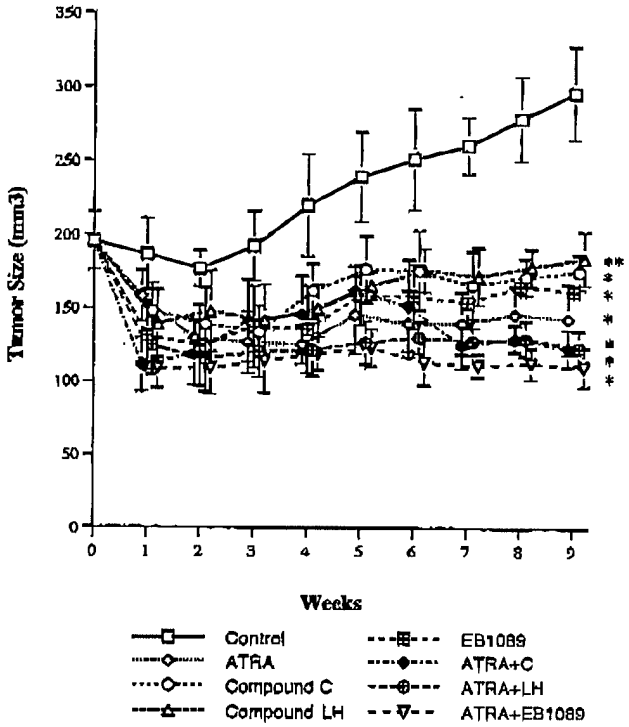

Figure 1. *Size of MCF-7 human breast tumors in mice receiving a vitamin D_3 analog and ATRA. Size (mm^3) of MCF-7 tumors in BNX mice during therapy with a vitamin D_3 analog and ATRA. MCF-7 human breast cancer cells (5×10^6) were injected subcutaneously; for nine weeks, a vitamin D_3 analog and ATRA were administered intraperitoneally to BNX mice. Tumor sizes were calculated as the product of the length, width and height (see Materials and Methods section) of each tumor; these measurements were done weekly. Data are expressed as the mean ± SD for six to ten tumors. Definitions: significantly different from control group at * $p < 0.002$, ** $p < 0.02$ as determined by Mann-Whitney U test.*

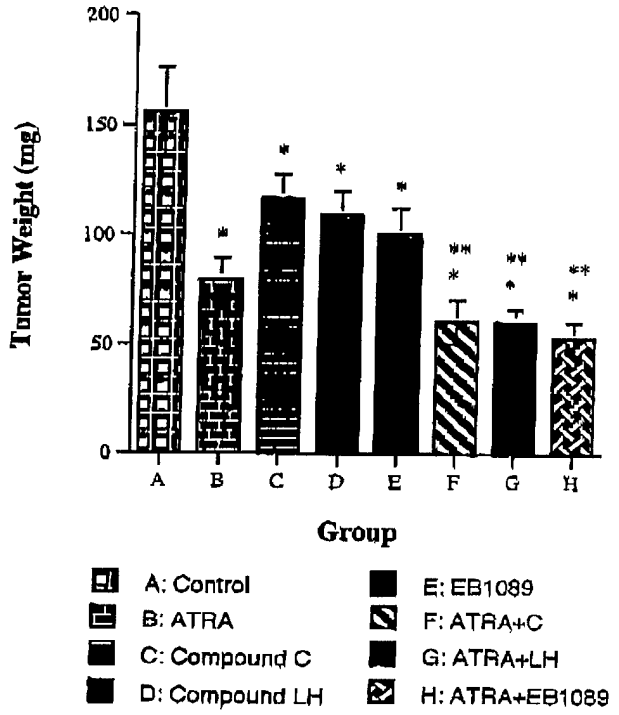

Figure 2. *Effect of a vitamin D_3 analog and ATRA either alone or in combination on the weight of MCF-7 human breast tumors in BNX mice. After nine weeks of treatments as described in the Materials and Methods and Figure 1, tumors were dissected and weighed. Results represent mean ± SD of six to ten tumors. * ($p<0.002$) and ** ($p<0.005$) represent the significant difference from the control group, and from the ATRA group, respectively as determined by Mann-Whitney U test.*

in combination (Figure 3D). Mice treated with the combination of compound LH and ATRA revealed approximately 25% necrosis and hyalinization, while mice treated with a combination of EB1089 plus ATRA revealed these changes in 40% of the tumor.

Table I shows the hematopoietic and blood chemistry data of the control and experimental mice at the end of the study. The doses of the vitamin D_3 analogs that caused a remarkable inhibition of size and weight of breast cancers did not elevate the levels of the serum calcium. White blood counts, hemoglobins, and hematocrits of ATRA + EB1089 treated group was slightly higher than that of the control cohort. All the ATRA treated groups had higher platelet counts compared to the control mice, but all the values were within normal range. No differences in the mean blood chemistry values were observed between the treated and untreated animals.

During the study, all mice were weighed once per week (Figure 4). All the treated groups had slightly lower body weights than the non-treated group. Nevertheless, the body weights of the treated groups were 93-99% of those in the control cohort. In general, each of the mice looked healthy.

Discussion

The present data show that the vitamin D_3 analogs and ATRA had potent anti-breast cancer activity *in vivo* without causing hypercalcemia and other major side-effects. Most importantly, combined treatment of the MCF-7 human breast cancer cells resulted in a stronger growth inhibition than treatment with either a vitamin D_3 compound or ATRA alone. Nevertheless, the combined therapy was not associated with any detectable toxicity.

$1,25(OH)_2D_3$ and its analogs can inhibit tumor growth by a variety of mechanisms, including regulation of angiogenesis (32), tumor invasiveness (33), apoptosis, and G_0/G_1, cell cycle arrest as a result in part of the enhanced expression of the cyclin dependent kinase inhibitors known as $p21^{waf1}$ and $p27^{kip1}$ (30,35-39). Despite promising antitumor activity of $1,25(OH)_2D_3$ *in vitro*, its calcemic toxicity *in vivo* limits the doses of the compound that can be given. The vitamin D_3 analogs, LH and EB1089, are both almost 50- to 100-fold

Table I. *Hematopoietic and Chemistry data of BNX mice at the end of therapy.*

	Control	ATRA	C	LH	EB1089	ATRA+C	ATRA+LH	ATRA+EB1089
WBC (10^3/mm^3)	1.92 ± 0.38	2.66 ± 0.56	1.83 ± 0.40	1.75 ± 0.68	2.40 ± 0.61	2.47 ± 1.24	1.50 ± 0.28	2.90 ± 2.12
RBC (10^6/mm^3)	8.61 ± 0.74	9.56 ± 0.53	8.69 ± 0.48	9.12 ± 0.53	8.61 ± 0.63	8.77 ± 0.82	8.79 ± 0.98	10.1 ± 2.24
Hgb (g/dl)	12.9 ± 0.99	13.7 ± 0.36	13.2 ± 0.67	14.0 ± 0.61	12.6 ± 0.83	12.6 ± 0.55	12.8 ± 2.26	15.0 ± 2.61
Hct (%)	42.4 ± 2.98	45.7 ± 2.10	41.9 ± 2.44	44.6 ± 1.93	40.0 ± 2.95	41.1 ± 1.62	42.0 ± 7.21	49.6 ± 9.55
Plt (10^3/mm^3)	913 ± 114	1293 ± 331	997 ± 82	1021 ± 96	890 ± 76	1135 ± 169	1419 ± 137	1347 ± 214

	Control	ATRA	C	LH	EB1089	ATRA ± C	ATRA ± LH	ATRA ± EB1089
GOT (U/L)	145 ± 31	126 ± 38	118 ± 55	109 ± 19	170 ± 69	99 ± 13	190 ± 56	112 ± 34
GPT (U/L)	92 ± 33	95 ± 31	78 ± 42	49 ± 21	86 ± 52	41 ± 11	84 ± 34	50 ± 9
BUN (mg/dl)	26.4 ± 3.1	22.7 ± 1.4	26.1 ± 1.3	22.4 ± 2.0	20.3 ± 1.7	18.0 ± 1.2	20.0 ± 2.1	16.9 ± 1.3
Cr (mg/dl)	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Bil (mg/dl)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
T-pro (g/dl)	5.1 ± 0.4	5.1 ± 0.6	5.0 ± 0.4	5.1 ± 0.3	5.0 ± 0.2	5.0 ± 0.2	4.9 ± 0.3	4.8 ± 0.4
Ca (mg/dl)	8.8 ± 0.4	9.1 ± 0.3	9.1 ± 0.4	8.9 ± 0.2	9.3 ± 0.3	8.8 ± 0.4	8.6 ± 0.4	8.7 ± 0.3

Abbreviations: C,compound C; LH,compound LH; ATRA, all-trans retinoic acid; WBC, white blood cell; RBC, red blood cell; Hgb, hemoglobin; Hct, hematocrit; Plt, platelet; GOT, glutamic oxalacetic transaminase; GPT, glutamic pyruvic transaminase; T-PRO, total protein; BUN, blood urea nitrogen; Cr, creatinine; Bil, bilirubin; Ca, calcium (normal range, 8.5-10.5 mg/dl).
The effect of intraperitoneal administration of vitamin D_3 analogs and ATRA on blood chemistry and hematopoietic parameters. All the vitamin D_3 analogs and ATRA were administerd intraperitoneally three times a week (M,W,F). Data are expressed as the mean ± SD for three to five mice.

more potent in their growth inhibitory activity as compared to compound C [1,25$(OH)_2D_3$], whereas *in vivo* studies have shown that the calcemic effects of the EB1089 analog was lower than 1,25$(OH)_2D_3$ (12,17,37-39). The compound LH has moderately higher calcemic activity than 1,25$(OH)_2D_3$ (34). Our data show that the tumor growth inhibitory action of the three vitamin D_3 compounds was statistical superior to the non-treatment group without causing any detectable toxicity including hypercalcemia.

Administration of ATRA has become established therapy for acute promyelocytic leukemia, and may have a role for treating solid tumors (1,21,40). Retinoid receptors have been demonstrated in breast and ovarian cancer cell lines, myeloma and leukemia cells (28,41-43). Synthetic retinoids have therapeutic effects on breast, ovarian, and prostate cancers in animal models (18-23). In this study, we report that the combination of one of the vitamin D_3 analogs with ATRA remarkably suppressed the growth of human breast cancer cells *in vivo* (Figure 2). This was most impressively shown when examining the tumor weight at the conclusion of the study. The tumor weights of the mice that received the combination of EB1089 with ATRA were approximately 70% less than those in the control cohort.

The histological data showed that all the MCF-7 breast cancer tumors were poorly differentiated adenocarcinoma. Tumors treated with ATRA and vitamin D_3 analog revealed necrosis and fibrosis involving approximately 30% of the tumor mass. Combination of ATRA and vitamin D_3 analogs produced approximately 40% necrosis and hyalinization of the tumors. To our knowledge this is the first *in vivo* evidence that a vitamin D_3 analog and a retinoid have cooperative anticancer activities. This is important because these compounds mediate their antiproliferative effects through different receptors and have non-overlapping toxicities.

Chemotherapy of breast cancer is based on the combined use of at least three major classes of anticancer drugs: alkylating agents, antimetabolites, and anthracycline antibiotics. Nevertheless, these combined chemotherapies are associated with toxicities and are not completely effective. Therefore, combinations of different forms of therapy including vitamin D_3 analogs with ATRA, as well as antiestrogens may be worthwhile especially as adjuvant therapy. Our data demonstrated that the combination of a vitamin D_3 analog and ATRA markedly inhibited the growth of human breast cancer cells *in vivo* without causing either hypercalcemia or other major side-effects. This combination has potential for the treatment of breast cancer patients.

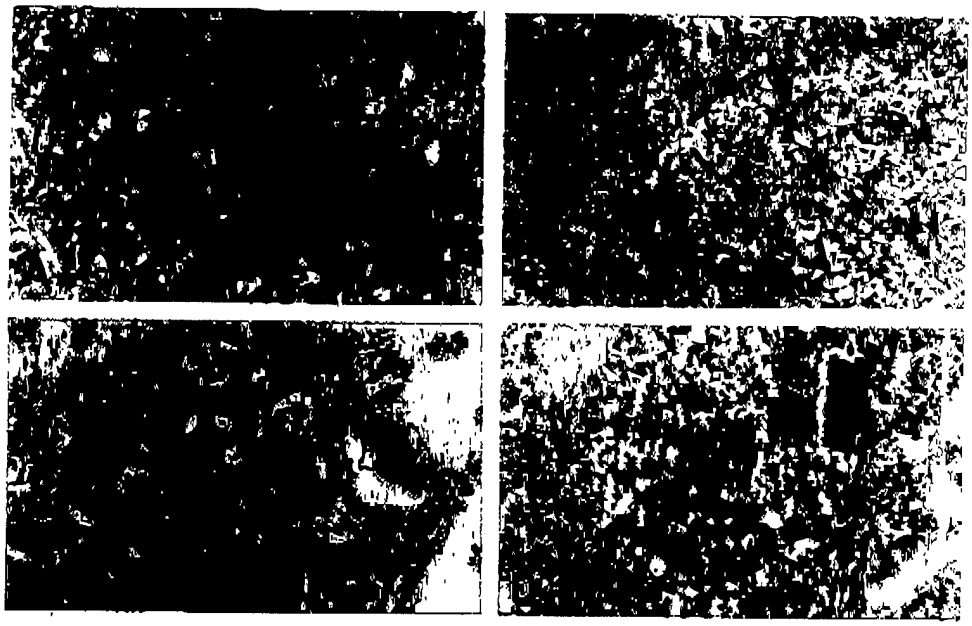

Figure 3. *Histological findings of tumors at the end of treatment. (Hematoxylin and Eosin, original magnification x 150).*
Figure 3A. *Control showing poorly differentiated infiltrating adenocarcinoma.*
Figure 3B. *Tumors treated with ATRA showing extensive necrosis with apoptosis.*
Figure 3C. *Tumors treated with ATRA displaying extensive fibrosis and hyalinization.*
Figure 3D. *Tumors treated with compound C and ATRA showing necrosis, apoptosis, and focal calcification (arrow).*

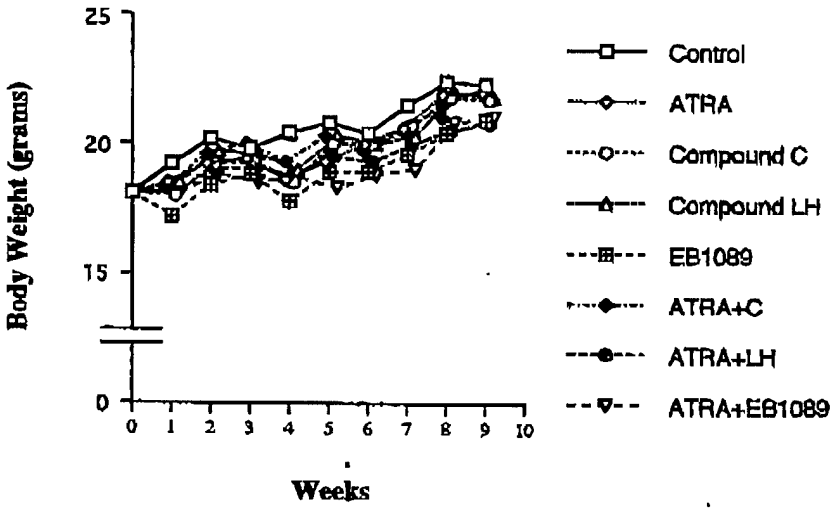

Figure 4. *Body weights of MCF-7 bearing mice receiving therapy. Results represent mean of three to five mice per group.*

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Report

Combined effect of vitamin D_3 analogs and paclitaxel on the growth of MCF-7 breast cancer cells *in vivo*

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Key words: breast cancer, BNX nude mouse, paclitaxel, Vitamin D_3 analogs

Summary

Vitamin D_3 analogs and paclitaxel (Taxol) are able to inhibit the *in vitro* growth of a variety of malignant cells including breast cancer cells. These two compounds decrease growth by different mechanisms and they have non-overlapping toxicities. We examined the abilities of three vitamin D_3 compounds to inhibit growth of a human mammary cancer (MCF-7) in BNX triple immunodeficient mice either alone or with Taxol. Vitamin D_3 analogs were $1,25(OH)_2D_3$ (code name, Compound C), $1,25(OH)_2$-16-ene-23-yne-19-nor-26,27-F_6-D_3 (Compound LH), and 24a,26a,27a,-trihomo-22,24-diene-$1,25(OH)_2D_3$ (EB1089). At the doses chosen, the antitumor effect of vitamin D_3 analogs alone was greater than that of Taxol alone, and an additive effect was observed when a vitamin D_3 analog and Taxol were administered together. EB1089 was the most potent compound, and the EB1089 plus Taxol was the most active combination, decreasing the tumor mass nearly 4-fold compared to controls. Weight-gain in each of the experimental cohorts at the end of the study was less than the control group, but the gain was significantly less in only two experimental groups (those receiving either EB1089 or Compound C plus Taxol). None of the animals became hypercalcemic, and their complete blood counts, serum electrolyte analyses, and liver and renal functions were all fairly similar and within the normal range. In summary, this combination of a vitamin D_3 analog and Taxol has the potential to be a therapy for breast cancer.

Abbreviations: $1,25(OH)_2D_3$: 1,25 dihydroxyvitamin D_3; VDR: vitamin D receptor; Taxol: paclitaxel

Introduction

Breast cancer is one of the most common malignant diseases of women in the United States, and it is associated with an appreciable morbidity and mortality. This continues to be true despite the introduction into clinical practice of several remarkable prognostic and therapeutic advances over the last two decades. Chemotherapy plays a central role in the treatment of breast cancer, either in the adjuvant setting or in those who fail endocrine manipulation.

The active vitamin D_3 metabolite, $1,25(OH)_2D_3$ (Compound C), is an important modulator of cellular proliferation and differentiation in a variety of normal and malignant cell types. An inverse correlation exists between annual exposure to sunlight and risk of breast cancer [1, 2]. Most breast cancer cell lines and more than 80% of breast tumors express high affinity intracellular vitamin D receptors (VDR) [3–6]. Patients whose breast cancer cells expressed VDR had significantly longer disease-free survival than those with receptor-negative tumors [7].

The hypercalcemic effect of $1,25(OH)_2D_3$ has prevented its application as a pharmaceutical agent. Recently, vitamin D_3 analogs have been developed that can inhibit cellular proliferation and induce differentiation without causing hypercalcemia [8–10]. Vitamin D_3 analogs decreased the development and progression of breast cancer and other carcinomas *in vivo* [8, 11], and inhibited metastatic spread of tumor cells [7].

The topically applied vitamin D_3 analog known as calcipotriol was able to inhibit the breast cancer tumor growth in patients with local skin recurrences [10]. One of the exciting new vitamin D_3 analogs identified to date is 1,25-$(OH)_2$-16-ene-23-yne-26,27-F_6-19-nor-D_3 (Compound LH) [12]. This potent vitamin D_3 analog reduced the development of breast cancer in nitroso-N-methylurea-treated rats [13]. Furthermore, dose-response studies showed that it was one of the most potent vitamin D_3 analog in suppressing clonogenic cancer growth, being able to suppress at 10^{-11} M greater than 50% clonal proliferation of the MCF-7 and SK-BR-3 breast cancer cells. The analog increased the proportion of cancer cells in G_0/G_1 phases and decreased those in the S phase of the cell cycle [12]. Pulse-exposure studies showed that three day exposure to LH (10^{-7} M) in liquid culture was able to achieve a 50% inhibition of subsequent MCF-7 clonal growth in soft-agar in the absence of analog, suggesting that inhibition of growth mediated by Compound LH is irreversible [12]. Further studies have found that the cyclin dependent kinase inhibitor known as $p27^{Kip1}$ is induced at high levels by Compound LH in the MCF-7 and SK-BR-3 breast cancer cells [12].

The analog 24a,26a,27a-trihomo-22,24-diene-1,25 $(OH)_2D_3$ (Compound EB1089) has a wide spectrum of anticancer activities *in vitro* including breast cancer cells [14–17]; the analog is 7 to 50-fold more potent than the parental 1,25$(OH)_2D_3$ (Compound C) *in vitro* against cancer cells. It appears to have similar affects on serum calcium levels as compared to 1,25$(OH)_2D_3$ [14]. A Phase I study in patients with advanced breast cancer showed that EB1089 can be given with predictable affects on cancer metabolism [17]. A Phase II trial of this novel vitamin D_3 analog is currently under way in patients with breast carcinoma.

The taxanes are an important new class of anticancer agents that exert their cytotoxic effects through a unique mechanism. Paclitaxel (Taxol) stabilizes microtubules and inhibits their depolymerization to free tubulin. It can block mitosis, induce extensive formation of microtubule bundles in cells, and cause multinucleation of cells during interphase [18–20]. Taxol, the first taxane in clinical trials, is active against a broad range of cancers that are generally considered to be refractory to conventional chemotherapy. This has led to the regulatory approval of Taxol in many countries for use as palliative therapy of patients with ovarian and breast cancers resistant to chemotherapy. Taxol was discovered as part of a National Cancer Institute program in which extracts of thousands of plants were screened for anticancer activity [18]. Taxol-based combinations, especially those with doxorubicin or cisplatin, appear promising for further study [21, 22].

The vitamin D_3 compounds and taxol have non-overlapping toxicities. To our knowledge, no one has studied the ability of the combination of a vitamin D_3 analog and Taxol to inhibit growth of human breast cancer *in vivo*. We report that this combination of therapy is efficacious in inhibiting the growth of MCF-7 tumors in BNX nude mice.

Materials and methods

Chemicals

The 1,25$(OH)_2D_3$ (Compound C) and 1,25$(OH)_2$-16-ene-23-yne-19-nor-26,27-F_6-D_3 (Compound LH) were synthesized by Milan Uskokovic at Hoffmann-LaRoche Inc. (Nutley, NJ) and 24a,26a,27a,-trihomo-22,24-diene-1,25$(OH)_2D_3$ (EB1089) was made by LEO Pharmaceutical Products (Ballerup, Denmark). Compounds C and LH were dissolved in absolute ethanol at 10^{-3}M as a stock solution, which was stored at 20°C and protected from light. EB1089 was dissolved in propylene glycol: 0.05M Na_2HPO_4 (80:20) at 0.1 mg/ml as a stock solution, which was stored at below 5°C and protected from light. Taxol (Bristol Myers, Squibb, NJ) was supplied as a concentrated sterile solution (6 mg/ml in a 17 ml ampule) in 50% polyoxyethylated Castor oil (Cremophor, EL), and 50% dehydrated alcohol. The drug was further diluted in PBS before administration.

Cell line

MCF-7 human breast cancer cells were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured according to their recommendations in culture flasks with vented filter caps (Costar, Cambridge, MA).

Mice

Forty female BNX nu/nu nude mice at 8 weeks of age were purchased from Harlan Sprague Dawley Inc. Indianapolis, IN USA, and maintained in pathogen-free conditions with irradiated chow.

Treatment protocol

Animals were bilaterally, subcutaneously injected with 10^6 MCF-7 cells/tumor in 0.1 ml Matrigel (Collaborative Biomedical Products, Bedford, MA). Before

Table 1. MCF-7 tumor size index in BNX mice a receiving Taxol and vitamin D_3 analogs

Treatment	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	9 weeks
A(control)	2.8 ± 1.4	7.6 ± 3.1	10.9 ± 3.9	11.6 ± 1.3	16.0 ± 3.0	96.3 ± 28.2	139.0 ± 21.4	170.8 ± 21.3
B(T)	2.8 ± 1.4	6.5 ± 2.1	9.5 ± 3.0	10.0 ± 3.5	13.6 ± 5.6	107.2 ± 22.8	125.5 ± 20.4	$131.0 \pm 15/8^{**}$
C(C)	2.7 ± 1.2	5.6 ± 2.4	8.2 ± 3.3	9.8 ± 3.3	13.8 ± 5.0	77.2 ± 28.7	$93.3 \pm 18.4^{**}$	$105.6 \pm 17.5^{**}$
D(C + T)	3.7 ± 1.0	6.4 ± 3.7	$6.0 \pm 3.5^{*}$	$6.5 \pm 3.2^{**}$	$8.8 \pm 5.3^{**}$	56.9 ± 21.6	$80.5 \pm 13.1^{**}$	$91.1 \pm 26.1^{**}$
E(LH)	3.0 ± 1.3	7.2 ± 1.7	8.0 ± 3.3	9.2 ± 2.7	12.4 ± 3.4	58.0 ± 16.1	$83.4 \pm 19.2^{**}$	$92.8 \pm 18.8^{**}$
F(LH + T)	2.1 ± 0.8	$4.1 \pm 1.9^{*}$	$6.0 \pm 2.7^{**}$	$7.5 \pm 2.6^{**}$	$9.0 \pm 2.8^{**}$	57.0 ± 11.5	$71.0 \pm 11.7^{**}$	$83.6 \pm 8.1^{**}$
G(EB1089)	2.2 ± 1.1	6.1 ± 2.7	$7.3 \pm 2.9^{*}$	$7.8 \pm 2.5^{**}$	$10.4 \pm 4.0^{*}$	$47.0 \pm 16.8^{*}$	$61.1 \pm 9.4^{**}$	$67.0 \pm 13.2^{**}$
H(EB1089 + T)	2.5 ± 1.1	5.4 ± 3.9	7.5 ± 3.8	7.2 ± 3.1	9.0 ± 3.6	58.4 ± 29.0	$55.5 \pm 18.6^{**}$	$61.0 \pm 13.2^{**}$

T: Taxol; C: compound C; LH: compound LH; s.c.: subcutaneous.
Tumor size index (mm^3) of MCF-7 tumors in BNX nude mice during therapy with vitamin D_3 analogs and Taxol which were administered intraperitoneally in BNX nude mice. Tumor size index was calculated from the length, width, and height of each tumor on every week according to the formula described in the Material and methods. Data are expressed as the mean ± SD.
*,**, Significantly different from Group A with $^{*}p < 0.05$, $^{**}p < 0.01$ as determined by Mann–Whitney U-test.

injection of cells, the animals received 300 rads whole body irradiation. Mice were divided randomly into eight groups of five mice each: Group A: nontreatment (control); Group B: Taxol; Group C: Compound C; Group D: Compound C + Taxol; Group E: Compound LH; Group F: Compound LH + Taxol; Group G: EB1089; and Group H: EB1089 + Taxol.

Vitamin D_3 analogs were administered intraperitoneally every other day at the following doses: Compound C, 0.05 μg per mouse; Compound LH, 0.0125 μg per mouse; and EB1089, 0.05 μg per mouse. The doses were chosen after a series of initial experiments determined the highest dose of the vitamin D_3 that could be given without causing hypercalcemia. Taxol (25 mg/kg per mouse) was administered intraperitoneally once a week. The dose was chosen from the report of Kalechman et al. [22]. One day after tumor injections, mice were treated with either vitamin D_3 analogs alone, Taxol alone, or the combinaiton of a vitamin D_3 analog and Taxol. During the experiment, four mice died: one in the Compound C + Taxol group; one in the Compound LH + Taxol group; and two in the EB1089 + Taxol group. The cause of their deaths was unknown.

Tumors were measured every week with vernier calipers. Tumor size index was calculated by the formula: $a \times b \times c$, where a is the length and b is the width and c is the height in millimeters.

Serum calcium values were measured on days 20 and 68 by atomic absorption spectrophotometry (Perkin-Elmer 560) and a modification of the calcium *o*-cresolphthalein complexone complexometric reaction (Dupont Analyst Benchtop Chemistry System, Dade International).

At the end of the experiment, animals were killed by CO_2 asphyxiation and tumor weights were measured after careful resection, and blood was also collected from the orbital sinus for chemistry and blood analysis. Chemistries and blood analyses were measured by Dupont Analyst Benchtop Chemistry System, Dade International, Newark, DE and by Serono-Baker 9000 Diff, Biochem Immuno-Systems, Allentown, PA, respectively.

Statistical analysis

The statistical significances of the differences were analyzed by the non-parametric Mann–Whitney U-test. Each animal carried two tumors, and each tumor was considered an independent observation.

Results

Table 1 shows the effect of vitamin D_3 analogs and Taxol, either alone or in combination, on the size of MCF-7 tumors during nine weeks of therapy. The antitumor activities of therapy were noted for some of the groups by the 4th week of therapy. At completion of the trial, all the treatment groups had statistically significantly smaller tumors than the non-treated group. Administration of vitamin D_3 analogs alone remarkably suppressed the growth of the tumors. The most potent single agent was EB1089 (Group G). The second in potency was Compound LH (Group E), followed by Compound C. The antitumor effect of vitamin D_3 analogs appeared greater than that of Taxol and the enhanced activity was observed when vitamin D_3 analog

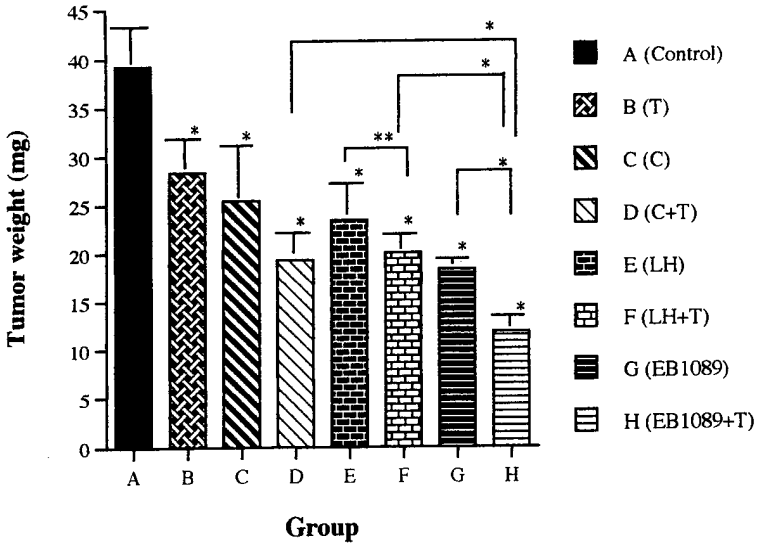

Figure 1. Effect of vitamin D_3 analogs and Taxol, either alone or in combination, on the weight of MCF-7 tumors in BNX nude mice. MCF-7 breast cancer tumors were established by subcutaneous injection of the cells. Vitamin D_3 analogs were administered intraperitoneally (i.p.) every other day except Saturday and Sunday. Taxol was administered i.p. once a week. After nine weeks of injections, tumors were dissected and weighed. Results represent means ± SD of six to ten tumors: a, significantly ($p < 0.01$) different from Group A. *, and ** represent data that are statistically significant at $p < 0.01$ and $p < 0.05$, respectively, as determined by Mann–Whitney U-test.

and Taxol were administered together. In each case, the combination of a vitamin D_3 compound and Taxol suppressed tumor growth more than either alone.

The results were similar when the effects of vitamin D_3 analogs and Taxol were evaluated by tumor weight at the conclusion of the study (Figure 1). Again, EB1089 was the most potent single agent, and the combination of a vitamin D_3 compound and Taxol was more potent than either alone. Tumor weights in the combined treatment groups were approximately 30–50% of those in the no treatment group. All the treatment groups were statistically different from Group A (control, $p < 0.01$). In addition, Group H (EB1089 + Taxol) was statistically different from Groups D (Compound C + Taxol) and F (Compound LH + Taxol).

Table 2 shows the serum calcium level of the control and experimental mice. The dose of these vitamin D_3 analogs that caused a remarkable inhibition of the size and weight of breast cancer did not elevate the level of the serum calcium (normal 8.5–10.5 mg/dl). We believe that initial calcium values were lower in all mice including controls than later in the study as a consequence of using two different methods of measurement. In the first measurement, we used atomic absorption spectrometry, and in the second we used a modification of the calcium *o*-cresolphthalein complexone complexometric reaction.

Table 2. The effect of vitamin D_3 analogs on serum calcium levels in BNX nude mice

	Days after initiation of treatment	
	20 days	68 days
A (control)	7.43 ± 0.42	9.34 ± 0.11
B (T)	7.41 ± 0.42	9.60 ± 0.17
C (C)	8.23 ± 0.34	9.08 ± 0.51
D (C + T)	8.46 ± 0.39	9.42 ± 0.15
E (LH)	8.92 ± 0.28	9.54 ± 0.36
F (LH + T)	8.99 ± 0.83	9.32 ± 0.22
G (EB1089)	8.53 ± 0.99	9.45 ± 0.12
H (EB1089 + T)	8.57 ± 0.41	9.22 ± 0.41

T: Taxol; C: compound C; LH: compound LH.
Normal murine serum calcium is 8.5–10.5 mg/dl.
The mice received intraperitoneal injections of vitamin D_3 analogs Monday, Wednesday, Friday and/or Taxol once per week. Blood was collected from the orbital sinus under anesthesia. Data are expressed as the mean ± SD.
Values are in mg/dl.

During the study, all mice were weighed once per week (Figure 2). Each of the cohorts gained in weight, but groups D (Compound C + Taxol) and G (EB1089) had statistically lower body weights than the non-treated group. The body weights in these three groups were 82–87% of that in the control Group A. The body weights of the other cohorts were not statistically dif-

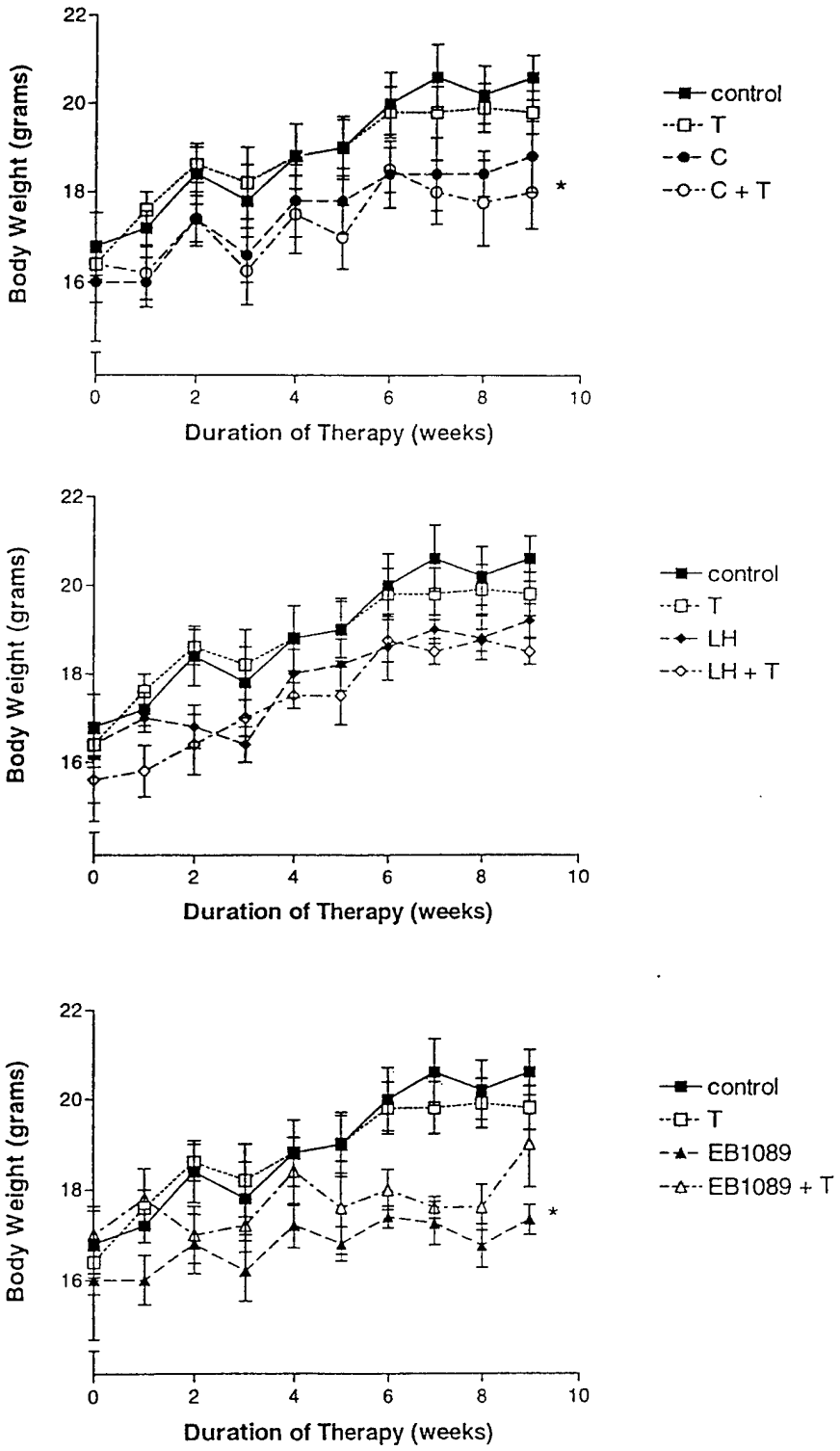

Figure 2. Body weight of MCF-7 bearing mice receiving therapy. Results represent means ± SD. *: $p < 0.05$ as determined by Mann–Whitney U-test.

Table 3a. Chemistry data of BNX mice at the end of therapy

	A (control)	B (T)	C (C)	D (C+T)	E (LH)	F (LH+T)	G (EB1089)	H (EB1089+T)
ALP (U/L)	153.4 ± 15.9	116.2 ± 14.7	164.4 ± 25.9	143.8 ± 12.6	142.8 ± 17.4	109.8 ± 22.8	158.3 ± 17.6	153.2 ± 41.6
GOT (U/L)	117.4 ± 38.2	181.0 ± 79.7	189.0 ± 50.3	183.5 ± 17.4	204.8 ± 57.3	152.8 ± 34.5	146.3 ± 24.2	158.0 ± 30.8
GPT (U/L)	65.6 ± 26.7	65.5 ± 22.1	62.8 ± 12.8	66.3 ± 11.1	95.8 ± 34.6	51.2 ± 8.0	49.7 ± 9.1	76.5 ± 20.1
T-PRO (g/dl)	4.6 ± 0.26	4.5 ± 0.36	4.5 ± 0.15	4.9 ± 0.06	4.7 ± 0.47	4.5 ± 0.35	4.4 ± 0.53	4.4 ± 0.48
BUN (mg/dl)	22.3 ± 1.41	9.5 ± 2.0	18.4 ± 1.0	16.0 ± 0.3	19.8 ± 0.9	20.4 ± 0.5	18.9 ± 0.8	17.4 ± 1.0
Cr (mg/dl)	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
Bil (mg/dl)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
UA (mg/dl)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	–
Glu (mg/dl)	220.6 ± 26.4	228.2 ± 13.7	205.4 ± 17.8	219.3 ± 30.5	198.6 ± 22.9	208.3 ± 18.3	200.0 ± 8.0	229.2 ± 23.3
Chol (mg/dl)	79.2 ± 89	73.0 ± 8.6	75.0 ± 13.3	70.3 ± 14.7	78.2 ± 5.2	75.8 ± 13.6	67.7 ± 5.9	92.0 ± 9.7
TG (mg/dl)	104.4 ± 22.5	84.4 ± 4.6	89.2 ± 9.9	89.3 ± 16.9	91.4 ± 6.8	81.8 ± 10.9	67.3 ± 14.2	76.4 ± 20.6

T: Taxol; C: compound C; LH: compound LH; ALP: alkaline phosphatase; GOT: glutamic oxalacetic transaminase; GPT: glutamic pyruvic transaminase; T-PRO: total protein; BUN: blood urea nitrogen; Cr: creatinine; bil: bilirubin; UA: uric acid; glu: glucose; Chol: cholesterol; TG: triglyceride data are espressed as the mean ± SD.

Table 3b. Hematopoietic data of BNX mice at the end of therapy

	A (control)	B (T)	C (C)	D (C+T)	E (LH)	F (LH+T)	G (EB1089)	H (EB1089+T)
WBC ($\times 10^3/\mu$l)	1.68 ± 0.26	1.63 ± 0.39	1.94 ± 0.57	1.60 ± 0.29	1.98 ± 0.22	1.68 ± 0.54	1.83 ± 0.25	2.90 ± 1.00
RBC ($\times 10^6/\mu$l)	8.90 ± 0.59	10.41 ± 0.91	9.0 ± 0.42	9.59 ± 0.39	8.83 ± 1.82	9.11 ± 0.55	8.44 ± 0.20	9.57 ± 0.77
Hgb (g/dl)	13.9 ± 0.92	15.4 ± 0.74	13.7 ± 0.61	14.5 ± 0.57	13.5 ± 2.25	14.3 ± 0.76	13.3 ± 0.61	15.2 ± 1.22
Hct (%)	42.2 ± 2.94	47.8 ± 2.80	42.5 ± 2.36	44.9 ± 2.13	41.8 ± 7.46	43.9 ± 2.44	41.8 ± 1.26	47.8 ± 4.29
MCV (picograms)	15.7 ± 0.48	14.8 ± 0.76	15.3 ± 0.56	15.2 ± 0.19	15.3 ± 0.74	15.8 ± 0.29	15.0 ± 0.25	15.9 ± 0.92
MCHC (%)	33.0 ± 0.48	32.2 ± 0.48	32.2 ± 0.52	32.4 ± 0.31	32.3 ± 0.55	32.7 ± 0.51	31.6 ± 0.47	31.9 ± 0.63
Plt ($\times 10^3/\mu$l)	940 ± 88.0	1014 ± 84.4	832 ± 97.6	945 ± 65.1	1048 ± 135	910 ± 22.4	895 ± 64.0	1131 ± 115.7

T: Taxol; C: compound C; LH: compound LH; WBC: white blood cell; RBC: red blood cell; Hgb: hemoglobin; Hct: hematocrit; MCV: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; Plt: platelet.
The effect of intraperitoneal administration of vitamin D_3 analogs and Taxol on blood analysis and chemistry. Vitamin D_3 analogs were administered intraperitoneally three times a week (M, W, F). Taxol was administered intraperitoneally once a week. Data are expressed as the mean ± SD for three to five mice.

ferent compared to the untreated control Group A. In general, each of the mice looked healthy.

The blood chemistry and hematopoietic data at the end of the study are presented on Table 3. Blood was collected from the orbital sinus while the animal was anesthetized. No difference in the mean blood chemistry and hematopoietic values were observed between the treated and untreated animals.

Discussion

The present data show that the vitamin D_3 analogs and Taxol had potent anti-breast cancer activity *in vivo* without causing hypercalcemia and other major side-effects. Combined treatment of the MCF-7 human breast cancer cells resulted in a stronger inhibition than treatment with either a vitamin D_3 compound or Taxol alone.

Antiestrogen therapy is the pivotal endocrine therapy of breast cancer [23]. However, breast cancer patients whose tumors do not express estrogen receptors constitute 30–40% of breast cancer patients [7], and they have a significantly worse prognosis than those with estrogen receptors [24]. Furthermore, resistance to antiestrogen therapy frequently occurs [25]; in these situations, treatment with vitamin D_3 might be useful.

The $1,25(OH)_2D_3$ and its analogs can inhibit tumor growth by a variety of mechanisms, including regulation of angiogenesis, apoptosis, tumor invasiveness, and G_0/G_1 cell cycle arrest as a result in part of the

enhanced expression of the cyclin dependent kinase inhibitors known as $p21^{WAF1}$ and $p27^{Kip1}$ [26–30]. Despite promising antitumor activity of $1,25(OH)_2D_3$ *in vitro*, its calcemic toxicity *in vivo* limits the doses that can be given. The vitamin D_3 analogs LH and EB1089 have almost the same growth inhibitory action as Compound C, but 50–100-fold lower concentrations of these analogs were required for this anticancer activity. In contrast, *in vivo* studies have shown that the calcemic activity of the EB1089 analog was lower than $1,25(OH)_2D_3$ [14, 31–33]. The Compound LH had slightly higher calcemic activity than $1,25(OH)_2D_3$ [14]. Our data show that the growth inhibitory action of the three vitamin D_3 compounds was statistically greater than that of the non-treatment group. Moreover, each of these cohorts had inhibition of tumor growth without hypercalcemia.

Taxol is one of the most important new cytotoxic agents to be introduced for the management of breast cancer in several years [18–22]. Combinations of Taxol with various cytotoxic agents are being actively explored [34–37]. As expected, the present data show that Taxol has an anti-breast cancer effect *in vivo*. The combination of one of the vitamin D_3 compounds with Taxol remarkably suppressed the growth of human breast cancer cells *in vivo* (Figure 1). This was shown most impressively when examining the tumor weights at the conclusion of the study, which decreased 70% in the mice that received the combination of EB1089 with Taxol as compared to that in the diluant-treated control group.

Chemotherapy of many stages of breast cancer is still based on the combined use of three major classes of anticancer drugs: alkylating agents, antimetabolites, and anthracycline antibiotics. Nevertheless, these combined chemotherapies are associated with overlapping toxicities and are not completely effective. Therefore, combinations of different forms of therapy including biologic modifiers such as vitamin D_3 analogs combined with Taxol, as well as antiestrogens, and retinoids may be worthwhile.

Taxol exerts its cytotoxic effects through a unique mechanism of microtubule stabilization resulting in blockade of mitosis [19, 20, 38]. The vitamin D_3 compounds are lipid soluble and freely enter the cell. They bind and activate the vitamin D_3 receptors, allowing efficient interaction with vitamin D response elements thus modulating the expression of various genes. Despite intense research, the exact mode of action by which vitamin $1,25(OH)_2D_3$ and its analogs inhibit cancer cell growth remains largely unknown [39]. They can lead to cell cycle arrest with elevation in levels of $p21^{WAF1}$ and $p27^{KIP1}$ cyclin dependent inhibitors. Taken together, the vitamin D_3 compounds and Taxol probably inhibit proliferation of cancer cells including those of the breast by different mechanisms. Furthermore, the toxicities of the two therapies are clearly different, with the vitamin D_3 compounds potentially producing hypercalcemia and the Taxols having the ability to cause hematopoietic cytopenias. Our data demonstrate that the combination of a vitamin D_3 analog and Taxol markedly inhibited the growth of human breast cancer cells (MCF-7) *in vivo* without causing either hypercalcemia, hematopoietic cytopenias, or other major side-effects. This combination has the potential for treatment of breast cancer patients, especially in the adjuvant setting.

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20-Cyclopropyl-cholecalciferol Vitamin D_3 Analogs: A Unique Class of Potent Inhibitors of Proliferation of Human Prostate, Breast and Myeloid Leukemia Cell Lines*

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Abstract. *We have synthesized and studied the ability of a series of nine novel 1,25 dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] analogs to inhibit clonal growth of myeloid leukemic cells (HL,60), prostate (LNCaP, PC-3 and DU-145) and breast (MCF-7) cancers cells. DU -145 cells were actively resistant to compounds (cmpd) with all of these modifications, but when we removed C-19 (E, 1,25-Dihydroxy-23E-ene-26,27-hexafluoro-19-nor-20-cyclopropyl-cholecalciferol) an analog resulted that was inhibitory against all three prostate cell lines, breast and HL-60 cell lines. Further analysis showed that pulse exposure (3 days, 10^{-7}M) to this analog was enough to inhibit clonal growth of PC-3 cell by 50%. Furthermore, cmpd E increased the number of PC-3 cells in G_1 and decreased the number in S phase. 1,25(OH)$_2$D$_3$ mediates its biological activities through specific binding to the vitamin D_3 receptor (VDR) and subsequent association with vitamin D_3 response elements (VDRE) in genes modulated by 1,25(OH)$_2$D$_3$. Several novel vitamin D_3 cmpds have recently been identified which have 5- to 1000-fold greater abilities to induce differentiation and to inhibit proliferation of prostate cancer, breast cancer and HL-60 leukemic blast cells as compared to the parental 1,25(OH)$_2$D$_3$. To clarify the mechanism by which nine of these vitamin D_3 analogs mediate their remarkably potent biological activities, we have investigated their abilities in PC-3 prostate cancer cells to transactivate a chroramphenicol acetyl transferase (CAT) reporter gene containing a VDRE from the human osteocalcin gene attached to a thymidine kinase minimal promoter. Dose-response studies of Cmpd E showed that in serumless culture conditions, transactivation of the VDRE-CAT was stronger than cmpd J [1,25(OH)$_2$D$_3$]. Then, we investigated the effects of vitamin D_3 cmpd J in mice. Our data showed the growth inhibitory action of the vitamin D_3 cmpd E in prostate cancer cell line (PC-3) was stastically superior to the non-treatment group in terms of tumor size and tumor weight in mice. In summary, this is the first report of a potent series of 20-cyclopropyl-cholecalciferol vitamin D_3 analogs with the ability to inhibit proliferation of LNCaP, PC-3, DU-145, MCF-7 and HL-60 cell lines. These cmpds may mediate their potent anti-proliferative activities through a cell cycle arrest pathway.*

Abbreviations used: 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; VDR, vitamin D$_3$ receptor; VDRE, vitamin D$_3$ response element; CAT, chloramphenicol acetyl transferase; compound, cmpd; A, 1,25-Dihydroxy-23-yne-20-cyclopropyl-cholecalciferol; B, 1,25-Dihydroxy-23-yne-26,27-hexafluoro-20-cyclopropyl-cholecalciferol; C, 1,25-Dihydroxy-23-yne-19-nor-20-cyclopropyl-cholecalciferol; D, 1,25-Dihydroxy-23-yne-26,27-hexafluoro-19-nor-20-cyclopropyl-cholecalciferol; E, 1,25-Dihydroxy-23E-ene-26,27-hexafluoro-19-nor-20-cyclopropyl-cholecalciferol; F, 3-Desoxy-1,25-dihydroxy-23-yne-20-cyclopropyl-cholecalciferol; G, 3-Desoxy-1,25-dihydroxy-23E-ene-26,27-hexafluoro-20-cyclopropyl-cholecalciferol; H, 1α-Fluoro-25-hydroxy-23-yne-20-cyclopropyl-cholecalciferol; I, 1α-Fluoro-25-hydroxy-23-yne-26,27-hexafluoro-20-cyclopropyl-cholecalciferol; J, 1,25 (OH)$_2$D$_3$; Waf, wild-type p53-activated fragment.

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Key Words: Vitamin D$_3$ analogs, inhibitors of proliferation, prostate cancer cell lines, CAT assay.

Prostate cancer has become the most frequently diagnosed non-skin cancer among American men, with an estimated 317,100 new cases in 1996 [1]. Despite the increase in the incidence of the disease and its large scale effects, no successful long-term therapies exist once the cancer progresses beyond the prostate capsule. Breast cancer is the most frequent cancer of women in the Western world. It responds to hormonal modulation as well as chemotherapy, but novel, nontoxic therapies are needed. Individuals with acute myeloid leukemia frequently can acheive remission, but

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they often relapse. Therapy during remission may be helpful in sustaining these remissions.

1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is a member of the seco-steroid family, and it can inhibit the growth *in vitro* of cancer cells from several different tissues, including human myeloid leukemia cells [2-5], breast [6-8], colon [9-11], squamous skin [12] cancers, and glioma cells [13]. In addition, the inhibition of proliferation of prostate primary malignant tissue and cell lines by 1,25$(OH)_2D_3$ has been demonstrated [14-19].

The chief side-effect of 1,25$(OH)_2D_3$ is hypercalcemia. Therefore, we have focused on developing new vitamin D_3 analogs with increased anticancer activity and decreased calcemic effects. The structure-function analysis of 1,25$(OH)_2D_3$ has centered on the four main motifs of the seco-steroid: the A ring, seco-B ring, C/D ring, and the side-chain. The addition of a cyclopropyl group at carbon 20 may enhance the antitumor activity of vitamin D_3 analogs. Therefore, we have synthesized and studied vitamin D_3 compounds (cmpds) that have a cyclopropyl moiety at carbon 20. To the cyclopropyl backbone, we also incorporated previously identified active structual motifs that are known to enhance activity, including desaturation at carbon (C)-23, addition of six fluorines at C-26 and -27, and removal of carbon 19. To our knowledge, this is the first report examining the activity of a cyclopropyl vitamin D_3 cmpd. A total of nine novel analogs having these structual changes were synthesized and tested for their ability to inhibit clonal growth of three prostate cancer cell lines (PC-3, DU-145, LNCaP), a breast cancer cell line (MCF-7) and an acute myeloid leukemia cell line, HL-60. One of the most potent analogs [1,25-dihydroxy-23E-ene-26,27-hexafluoro-19-nor-20-cyclopropyl-cholecalciferol] was studied in greater detail by determing its ability to inhibit clonal proliferation of LNCaP after pulse-exposure to the analog as well as its ability to modulate levels of the cyclin dependent kinase inhibitors (CDKIs) $p21^{waf1}$, $p27^{kip1}$, to transactivate a reporter gene containing a vitamin D_3 response element (VDRE) and to affect growth of human prostate cancer implanted in BNX nude mice.

Materials and Methods

Cell lines. The prostate cancer cell lines (LNCaP, PC-3 and DU-145), breast cancer cell line (MCF-7) and leukemic cell line (HL-60) were obtained from American Type Culture Collection (ATCL, Rockville, MD). The cells were cultured in either DMEM or RPMI 1640 (GIBCO Life Technologies, Grand Island, NY) 10% bovine fetal serum according to ATCC's recommendations, in culture flasks with vented filter caps (Costar, Cambridge, MA).

Vitamin D_3 compounds. The vitamin D_3 cmpds were dissolved in absolute ethanol at 10^{-3} M as stock solution, which were stored at -20°C and protected from light. The analogs [Code names: A, B, C, D, E, F, G, H, I, J] were synthesized by Hoffmann LaRoche Inc. (Nutley, NJ) (Figure 1).

Clonogenic assay in soft agar and pulse-exposure experiments. The cancer cells were cultured in a two-layer soft agar system for 14 days, as described previously [22]. For the pulse-exposure studies, the LNCaP cells were exposed to analog E [1,25-(CH)2-23E-ene-26,27-hexafluoro-19-nor-20-cyclopropyl-cholecalciferol] (10^{-7}M) for varicus durations, carefully washed twice, counted and plated into 24-well plates for soft agar colony assay.

Cell cycle analysis by flow cytometry. Cell cycle analysis was performed on prostate cancer cells incubated for 72h with or without cmpd E (10^{-7}M). The methanol-fixed cells were incubated for 30 min at 4°C in the dark with a solution of 50 µg/ml propidium iodide, 1 mg/ml Rnase(100 units/ml;Sigma Chemical Co.), and 0.1% NP40(Sigma). Analysis was performed immediately after staining using the CFLLFit program (Becton Dickinson), whereby the S-phase was calculated with a Rfit model.

Transfection and assay of CAT activity. The PC-3 cells were maintained in RPMI 1640 medium with 5% fetal calf serum (FCS). 5×10^5 cells were transfected by lipofection of 1.5 µg of pBLCAT2-VDRE3 plasmid [20,21,23] and 0.5 µg of CMV luciferase plasmid after the cells were washed with OPTI-MEM (GI300). The cells were cultured with cmpd E (10^{-11}-10^{-7}M) for 36 hours in serum-free conditions (Serumless Medium; GIBCO) and harvested and CAT lystates were prepared. CAT activity was assayed by thin-layer chromotagraphy and autoradiography.

Mice. Fifteen male BNX nude mice at 8 weeks of age were purchased from Harlen Sprague Dawley Inc. Indianapolis, NJ USA, and maintained in pathogen-free conditions with irradiated chow.

Treatment protocol of mice. Animals were bilaterally, s.c. injected with 3×10^6 PC-3 cells/tumor in 0.1 ml matrigel (Collaborative Biomedical Products, Bedford, MA). Before injection of cells, the animals recieved 300 rads whole body irradiation. Mice were divided randomly into 3 groups of 5 mice each: no treatment (control); Cmpd E_1; Cmpd E_2. The cmpd E was administered intraperitonealy every other day except Saturday and Sunday at the following doses: compound E_1, 0.005 µg/mouse, E_2, 0.01 µg/mouse. Mean doses were chosen after a series of initial experiments determined the highest dose of cmpd E that could be given without causing hypercalcemia. One day after tumor injection of PC-3 prostate cancer cells, mice were treated with cmpd E. Tumors were measured every week with vernier calipers. Tumor size were calculated by the formula: a × b × c, where a is the length, b is the width and c is the height in millimeters.

At the end of the experiment, animals were killed by CO_2 asphyxiation and tumor weights were measured after careful resection; blood was also collected from the orbital sinus for serum calcium. Serum calcium values were measured on day 42 by atomic absorption spectrophotometry (Perkin-Elmer 560).

Statistical analysis. Statistical significance of the difference was analyzed by the non-parametric Mann-Whitney U test.

Results

Effect of vitamin D_3 analogs on clonal proliferation of cancer cells from various tissues. The cancer cells were cloned in soft agar in the presence of vitamin D_3 analogs at 10^{-11} to 10^{-6} M. Dose-response curves were drawn (Figure 2) and the effective dose that inhibited 50% colony formation (ED_{50}) was determined (Table I). The 1,25 D_3 analogs were very potent in their inhibition of clonal proliferation of HL-60, MCF-7, PC-3 and LNCaP cell lines (Figure 2). Analog E [1,25-dihydroxy-23E-ene-26,27-hexafluoro-19-nor-20-cyclopropyl-cholecalciferol] was the most potent cmpd, achieving an ED_{50}

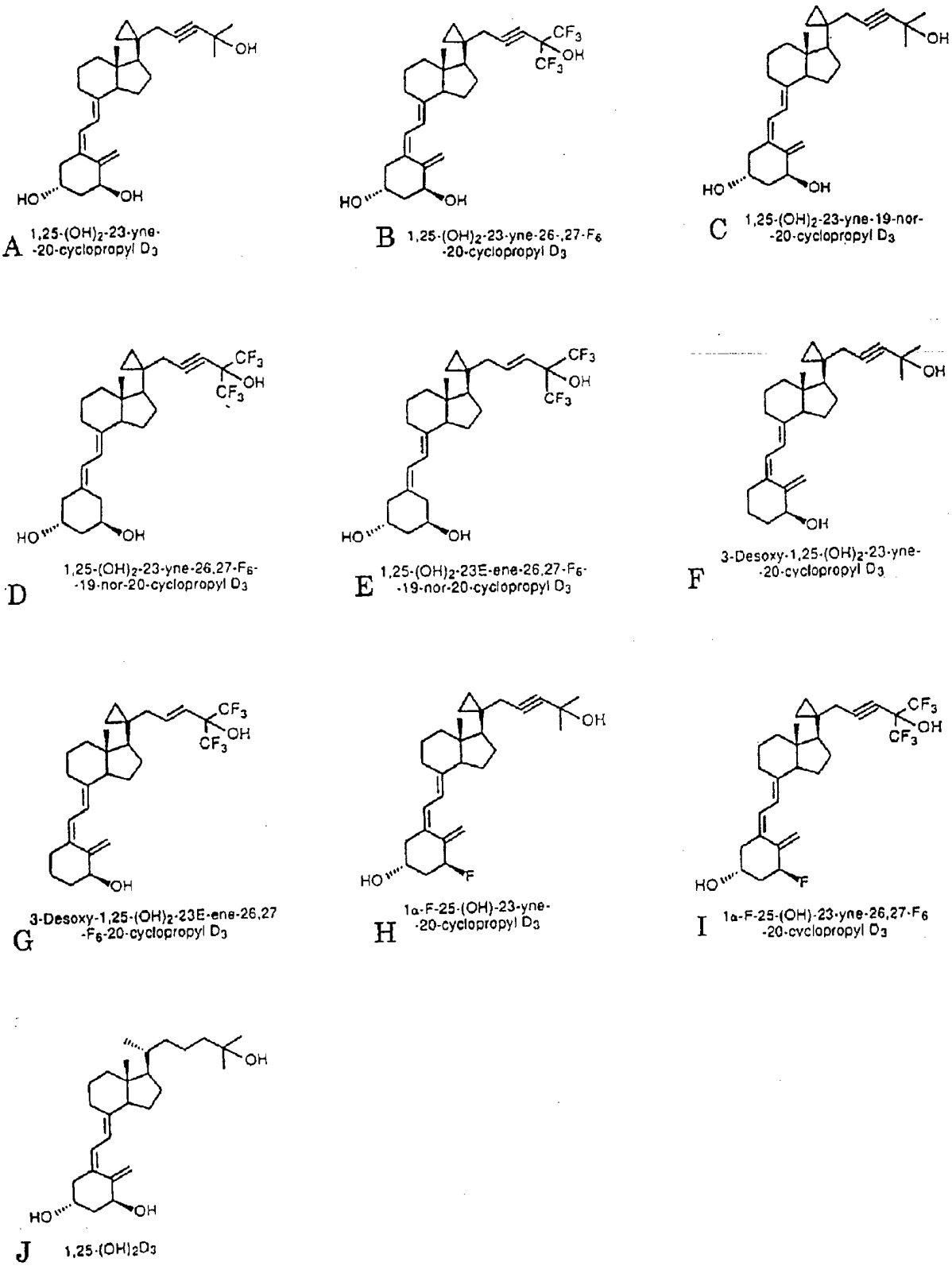

Figure 1. *Chemical structures and code names of novel vitamin D3 analogs.*

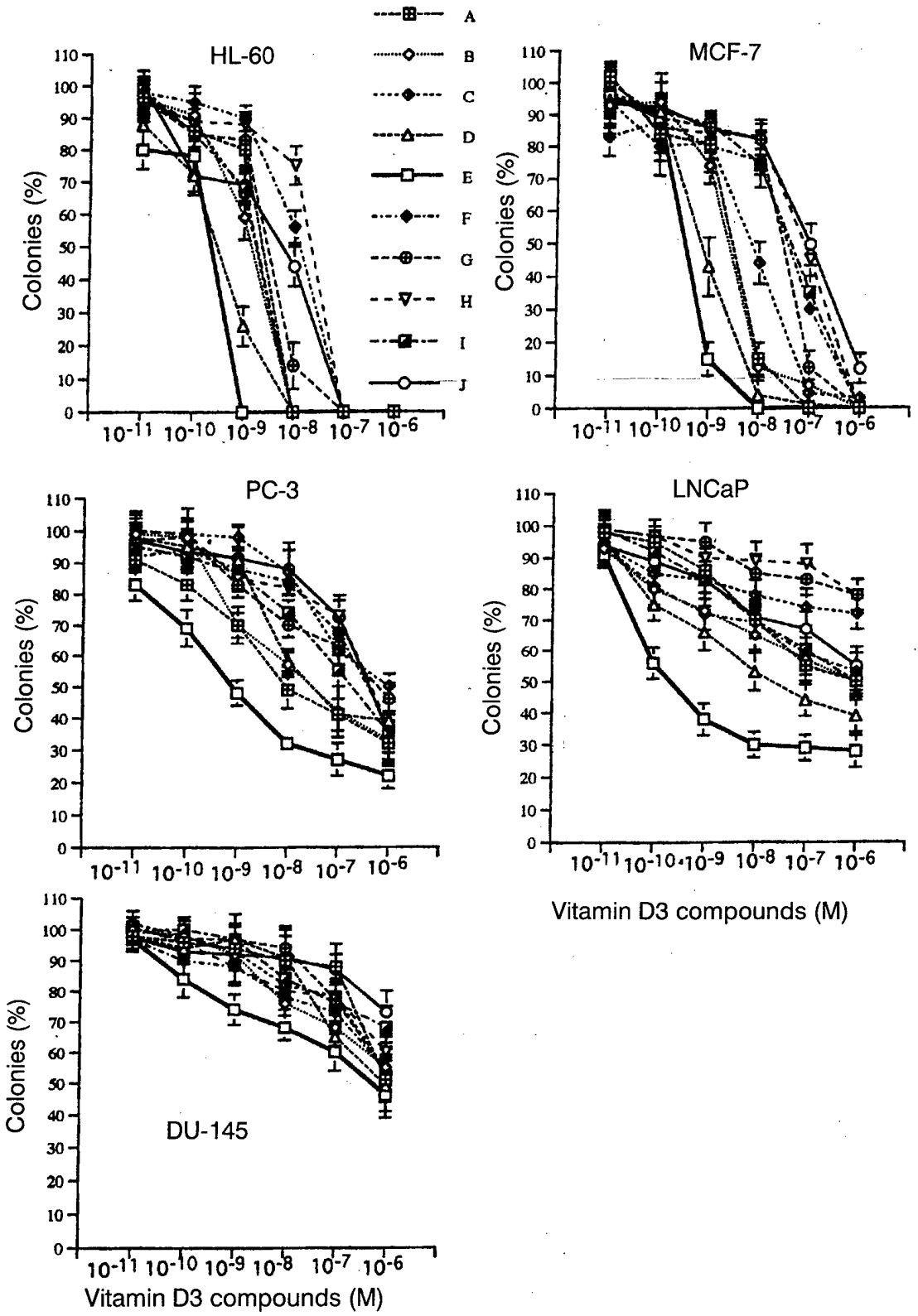

Figure 2. *Dose-response effects of vitamin D_3 compounds on clonal proliferation of several cancer cell lines. Results are expressed as a mean percentage ± SD of control plates containing no vitamin D_3 compounds. Results are the means of at least three independent experiments with triplicate dishes.*

Table I. *Inhibition of clonal proliferation of cancer cell lines by vitamin D_3 analogs.*

	Inhibition of clonal proliferation ED_{50} (M)[a]				
Vitamin D_3 analogs	HL-60	MCF-7	PC-3	LNCaP	DU-145
A	5×10^{-9}	5×10^{-9}	1×10^{-8}	1×10^{-6}	N.R.[b]
B	8×10^{-9}	4×10^{-9}	3×10^{-8}	N.R.	N.R.
C	5×10^{-9}	8×10^{-9}	1×10^{-6}	N.R.	N.R.
D	5×10^{-10}	8×10^{-10}	8×10^{-7}	3×10^{-8}	1×10^{-6}
E	2×10^{-10}	4×10^{-10}	9×10^{-10}	3×10^{-10}	8×10^{-7}
F	2×10^{-8}	6×10^{-8}	8×10^{-7}	N.R.	N.R.
G	5×10^{-9}	5×10^{-8}	8×10^{-7}	N.R.	N.R.
H	3×10^{-8}	8×10^{-8}	5×10^{-7}	N.R.	N.R.
I	6×10^{-9}	8×10^{-8}	2×10^{-7}	N.R.	N.R.
J [1,25(OH)$_2$D$_3$]	8×10^{-9}	1×10^{-7}	8×10^{-7}	N.R.	N.R.

[a] Dose-response curves (Figure 2) were used to calculate the concentration of the analogs achieving a 50% inhibition (ED_{50}) of clonal growth. N.R.,the ED_{50} was not reached at $\leq10^{-7}$ M of the 1,25 D_3 analog.

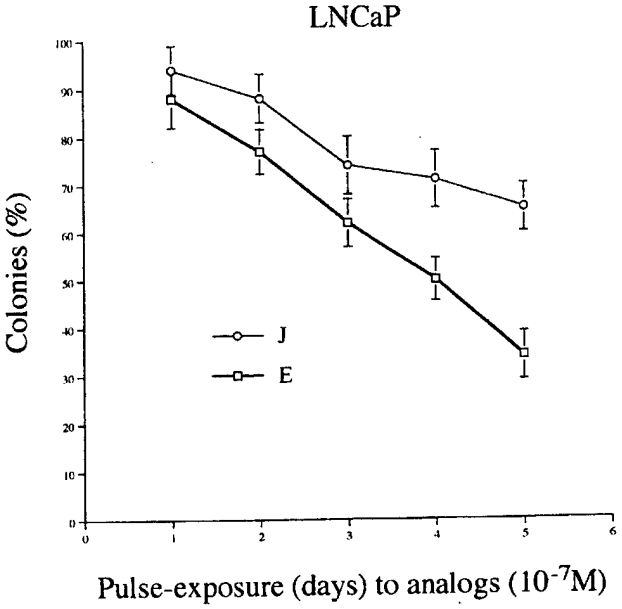

Figure 3. *Pulse-exposure of LNCaP prostate cancer cells to analog E. LNCaP cells were exposed for various durations to analog E (10^{-7}M). The cells were then thoroughly washed twice, plated into soft agar, and colonies were counted 14 days after plating. Results are the mean of 3 inndepedent experiments and are expressed as a mean percentage ± SD of control plates that received the same treatment but were not exposed to analog.*

of 2×10^{-10}, 9×10^{-10} and 3×10^{-10} M for HL,60, PC-3 and LNCaP cells, respectively (Table I). The DU-145 prostate cancer cells were less sensitive to the vitamin D_3. Because cmpd E was the most potent analog, all subsequent experiments focused on this analog.

Pulse exposure experiments. The LNCaP cells were exposed to analog E (10^{-7}M) for various durations, washed twice to remove the analog, plated in soft agar, and colony numbers enumerated on day 14 (Figure 3). Fifty per cent of the clonogenic cells were inhibited by 4 days of exposure to analog E, suggesting that this cmpd was capable of mediating a relatively rapid, irreversible inhibition of growth of these cells.

Cell cycle analysis. Effect of cmpd E on the cell cycle of prostate cancer cells was determined using LNCaP prostate cancer cells as a target. After a 72 hr exposure to cmpd E (10^{-7}M), a significant increase in the percent of these cells in the G_0/G_1 phase of the cell cycle [88% with cmpd E, 72% in control cells] occured with a concomitant decrease in the per cent of cells in S phase [6% with cmpd E, 22% in control cells] ($p<0.05$; Figure 4).

Transfection and assay of CAT activity. The 1,25(OH)$_2$D$_3$ response element (VDRE) was placed in front of a thymidine kinase promoter of the reporter gene CAT. This construct was transfected into PC-3 cells. In the absence of cmpd E, almost no CAT activity was detectable (Figure 5). Cmpd E increased CAT activity in a dose-response manner. At 10^{-7} mol/L, cmpd E increased CAT activity nearly 12.5 fold as compared with cells cultured in control media having no added vitamin D_3 (Figure 5A,B).

Effect of vitamin D_3 analog on the growth of PC-3 prostate tumors in mice. Administration of the vitamin D_3 analog E at two different doses (E_1, 0.005 μg and E_2, 0.01 μg) statistically significantly retarded the growth of the prostate cancer cells as compared to the diluant treated control group ($p<0.05$) (Figure 6A). The results were similar when the effect of the vitamin D_3 analog E was evaluated by tumor weights at the conclusion of the study ($p<0.05$)(Figure 6A). The vitamin D_3 analog E did not elevate the level of the serum calcium (normal 8.5-10.5 mg/dl). Tumors in the diluant-control mice showed infiltrating poorly differentiated adenocarcinoma (Hematoxylin and eosin x 200) (Figure 7A). Tumors from the mice that received cmpd E_2 (0.01 μg) were almost entirely necrotic with scattered nuclear fragments (apoptotic bodies) (Hematoxylin and eosin × 200) (Figure 7B).

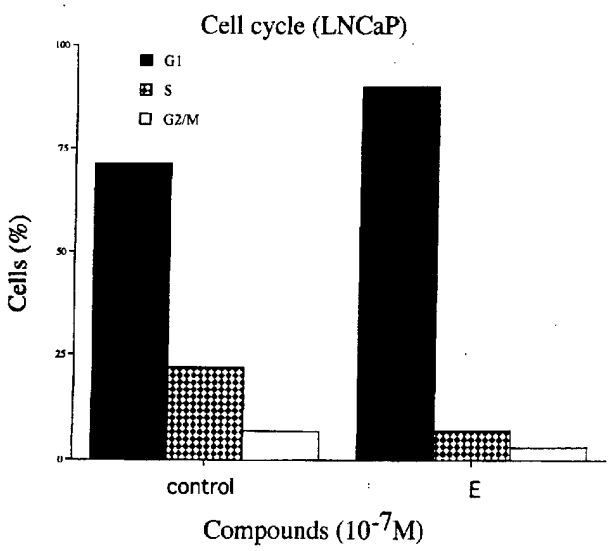

Figure 4. *Cell cycle analysis of breast cancer cells which had been cultured with either compound C [1,25(OH)$_2$D$_3$] or analog E at 10^{-7}M for 3 days. Control cells represent those not exposed to vitamin D3 compounds. Each point represents a mean of at least three independent experiments.*

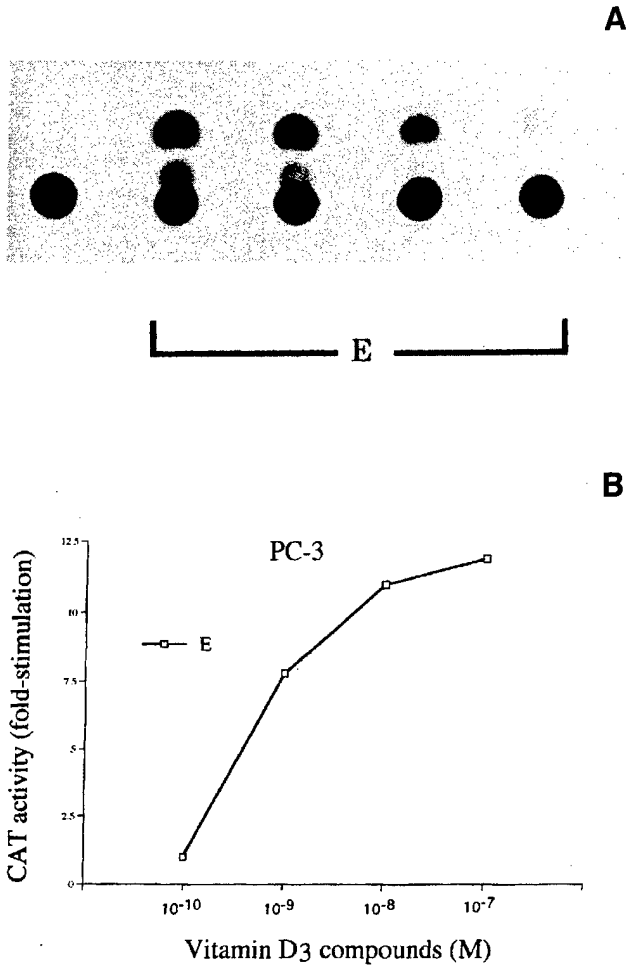

Figure 5. *Effect of 1,25-Dihydroxy-23E-ene-26,27-hexafluoro-20-cyclopropyl- l9 nor cholecalciferol (E) on transcriptional activation as measured by CAT assay in PC-3 prostate cancer cells. (A) A representative autoradiogram of CAT activity by thin-layer chromatography. 1st lane is control; Subsequent lanes represent 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M cmpdE, respectively. (B) Fold-enhancement of CAT activity by increasing concentrations of vitamin D$_3$ compound E.*

Discussion

The prostate and breast cancer and leukemic cell lines varied in their sensitivities to the clonal inhibitory effects of the various 1,25(OH)$_2$D$_3$ cmpds. Four of the 5 lines were inhibited by each of the analogs. The DU-145 cell line was more resistant to the antiproliferative activity of the 1,25(OH)$_2$D3 analogs. The cmpd E was the most active analog, it has double bonds at C23, hexafluoro substitutions at C26 and C27, lacks a methylene at C19 as well as having a 20-cyclopropyl moiety. Earlier, we showed that 1,25-(OH)$_2$-16-ene-23-yne-26,27-F$_6$ D$_3$ (cmpd LH) potently inhibited growth of prostate cancer cell lines [19], but we also showed that administration of this analog at 0.0125 μg I.P. three times per week caused severe hypercalcemia suggesting its therapeutic ratio (anticancer potency versus toxicity) was not favorable [5].

Pulse-exposure of PC-3 prostate cancer cells for 3 days to analog E (10^{-7}M), washing, plating in soft agar, and enumerating colony formation 14 days after plating resulted in 50% inhibition of colony formation. These results suggest that cmpd E inhibited growth of the cancer cells by a mechanism other than one that is merely cytostatic (Figure 3). Furthermore, cmpd E increased the percentage of PC-3 prostate cancer cells in G$_1$ and decreased the number of these cells in S phase (Figure 4). The 1,25(OH)$_2$D$_3$ has been associated with cell cycle arrest in G$_0$/G$_1$. Many factors can lead to a cell cycle arrest, but CDKIs p21^{wafl} and p27^{kipl} have been shown to be pivokal in this process [24,25]. Recently, investigators have shown that p21^{wafl} contains a VDRE within its promoter region [26]. Prior studies, found that several 1,25(OH)$_2$D$_3$ analogs (10^{-7}M,48h) caused a G$_1$ to S-phase block in parallel with an increased abundance of $p27^{kip1}$ in HL-60 cells, breast and prostate cancer lines [27,28]. We have found that the levels of $p21^{Waf1}$ and $p27^{Kip1}$ in LNCaP cell line increased by several fold after exposure to cmpd E (3 days, 10^{-7}M) (data not shown), supporting the hypothesis that the $p21^{Waf}$ and $p27^{Kip1}$ protein may be mediators of the anti-proliferative activity of the vitamin D3 cmpds by blocking entry of these cancer cells into the S-phase.

Our *in vivo* studies showed that cmpd E retarded the growth of PC-3 prostate tumors without inducing hypercalcemia. Previously, we found that 1,25(OH)$_2$D$_3$ at a dose of 0.0125 μg/mouse given in a similar time schedule caused mild hypercalcemia [5]. Why cmpd E was more potent

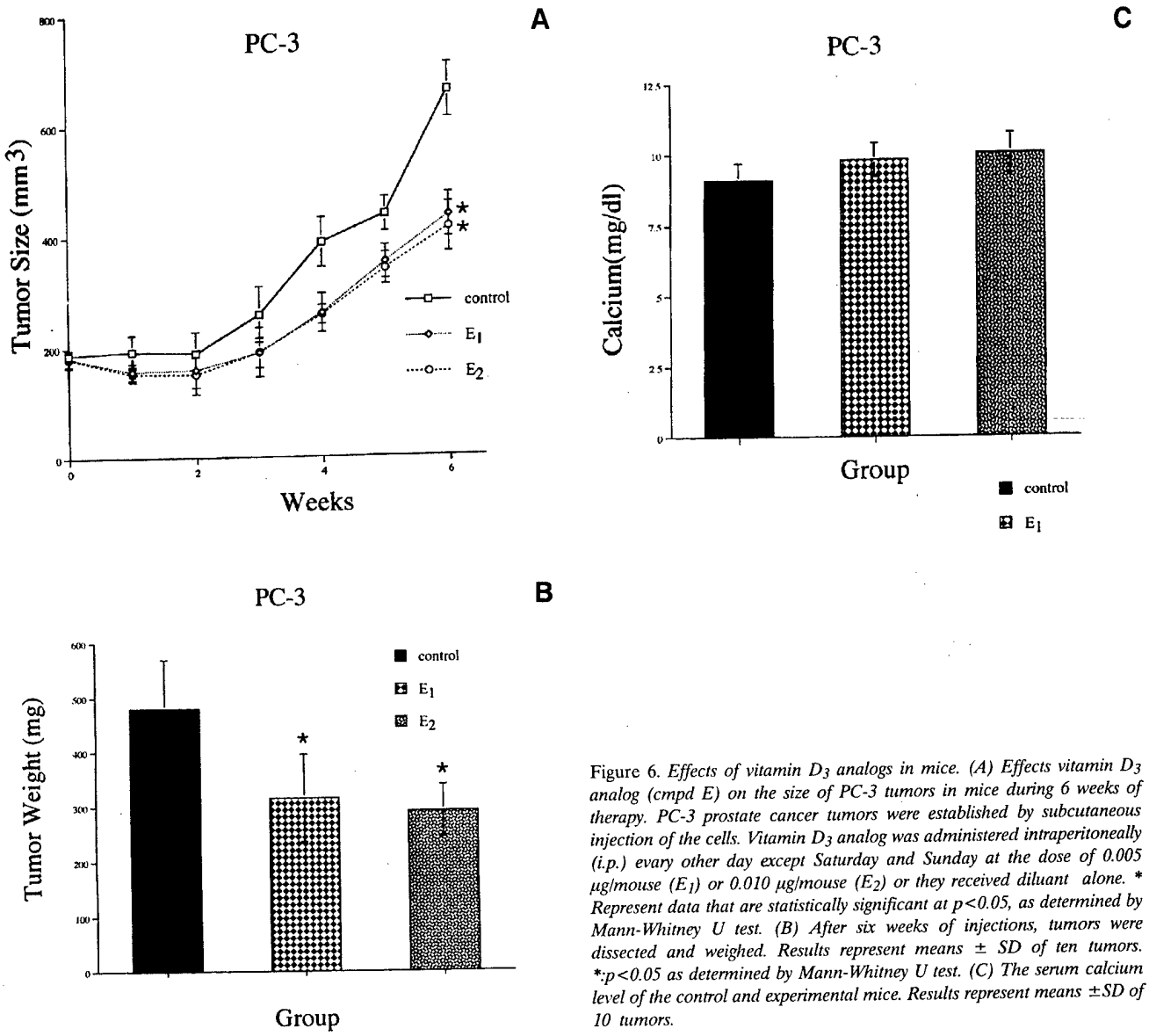

Figure 6. *Effects of vitamin D_3 analogs in mice. (A) Effects vitamin D_3 analog (cmpd E) on the size of PC-3 tumors in mice during 6 weeks of therapy. PC-3 prostate cancer tumors were established by subcutaneous injection of the cells. Vitamin D_3 analog was administered intraperitoneally (i.p.) evary other day except Saturday and Sunday at the dose of 0.005 μg/mouse (E_1) or 0.010 μg/mouse (E_2) or they received diluant alone. * Represent data that are statistically significant at $p<0.05$, as determined by Mann-Whitney U test. (B) After six weeks of injections, tumors were dissected and weighed. Results represent means ± SD of ten tumors. *:$p<0.05$ as determined by Mann-Whitney U test. (C) The serum calcium level of the control and experimental mice. Results represent means ±SD of 10 tumors.*

than $1,25(OH)_2D_3$ in its anti-cancer proliferative effects but did not cause more hypercalcemia than did $1,25(0H)_2D_3$, is not totally clear. Analogs may extend the half- life of the activated VDR [29] or they may induce novel VDR conformation [30], which may in turn allow either more efficient interaction with vitamin D_3 response elements and/or alter the array of vitamin D_3 response elements that can be activated. In our study, cmpd E increased VDRE directed reporter gene activity in a dose-response manner. At 10^{-7} mol/L, cmpd E increased CAT reporter gene activity nearly 12.5-fold compared with similar cells exposed to 10^{-10} mol/L of the same analog (Figure 5B).

Our data showed that the growth inhibitory action of cmpd E on the prostate cancer cell line (PC-3) growing in triple immunodeficient mice was statistically superior to the non-treatment group as measured both by tumor size and weight in mice. Use of hormones (*e.g.,* estrogen for prostate cancer) or hormone inhibitors (*e.g.,* tamoxifen for breast cancer and androgen inhibitors for prostate cancer) have become integral to the management of cancers. The new vitamin D_3 analogs may also assume a similar role in our armamentarium against selected cancers. In summary, we have identified a group of 1,25 D_3 analogs, including cmpd E, with potent anti-proliferative effects on three major cancers of man without discernible toxicities. Further studies in animals appear warranted in order to progress to clinical trials.

Acknowledgements

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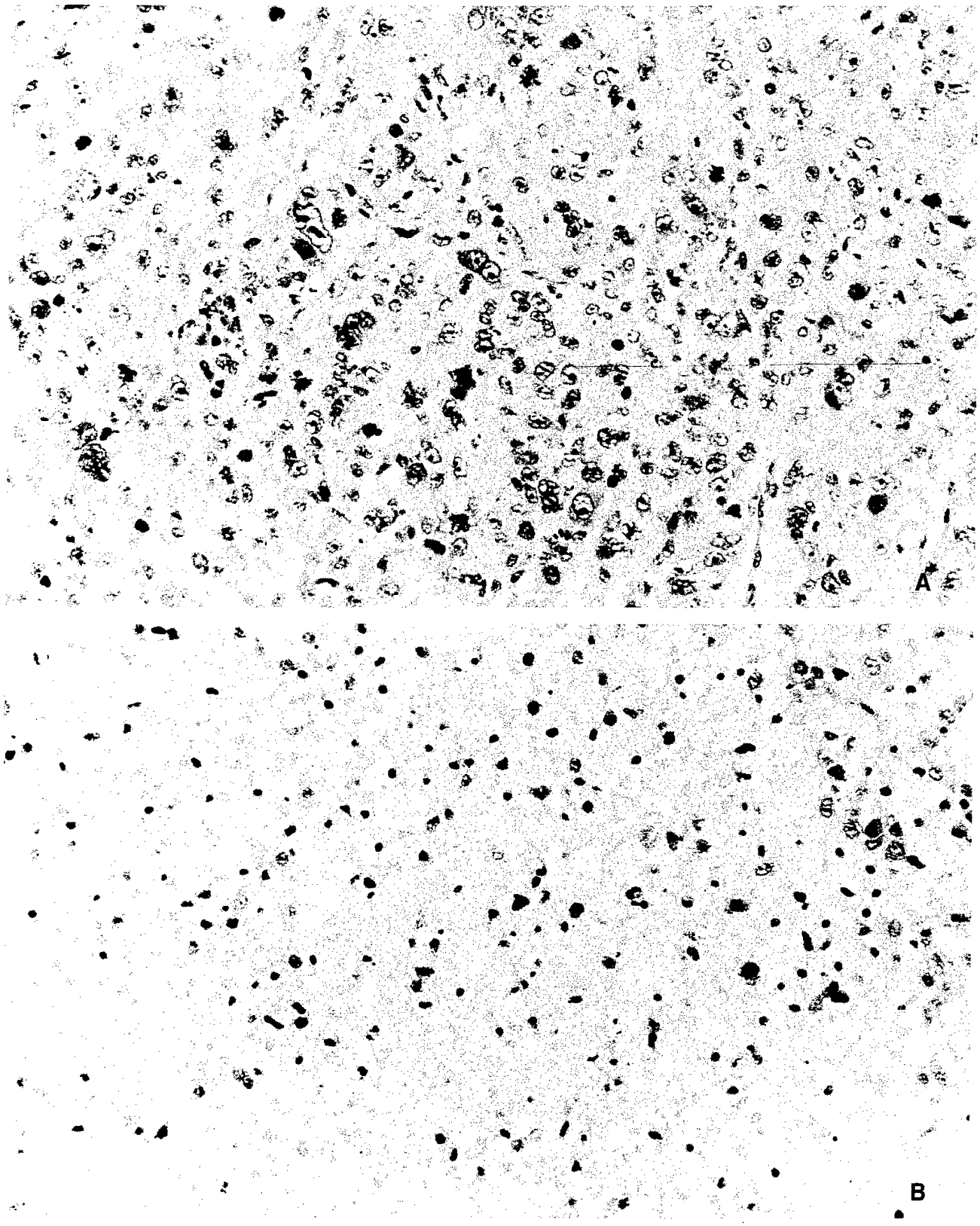

Figure 7. *Tumor histology in mice treated with either cmpd E_1 or diluant control. (A) Control tumor showing infiltrating poorly differentiated adenocarcinoma (Hematoxylin and eosin ×200). (B) Tumor treated with cmpd E_2 is almost entirely necrotic with scattered nuclear fragments (apoptotic bodies) (Hematoxylin and eosin x 200).*

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REPORT

A Ligand of Peroxisome Proliferator-Activated Receptor γ, Retinoids, and Prevention of Preneoplastic Mammary Lesions

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Background: **Chemoprevention of breast cancer is an active area of investigation. Recent *in vivo* and *in vitro* studies have shown that thiazolidinediones (e.g., troglitazone) and retinoids are able to inhibit the growth of breast cancer cells. Troglitazone mediates its action via peroxisome proliferator-activated receptor γ (PPARγ). We evaluated the ability of troglitazone, alone or in combination with retinoids, to prevent the induction of preneoplastic lesions by 7,12-dimethylbenz[*a*]anthracene (DMBA) in a mouse mammary gland organ culture model.** ***Methods:*** **Mammary glands of BALB/c mice were treated with DMBA (2 μg/mL) to induce preneoplastic lesions in organ culture. Effects of troglitazone, all-*trans*-retinoic acid (retinoic acid; ligand for retinoic acid receptor [RAR] α), and LG10068 (ligand for retinoid X receptors [RXRs]), singly or in combination, on the development of lesions were evaluated. Expression of retinoid receptors (RARα and RXRα) and PPARγ was determined by western blot analysis. Statistical significance was determined by generalized chi-squared analysis using the GENCAT software program and Bonferroni correction. All *P* values are two-sided.** ***Results:*** **Troglitazone (at 10^{-5} *M*) or retinoic acid (at 10^{-6} *M*) markedly inhibited the development of mammary lesions (both *P* values <.05); however, together they did not enhance the effectiveness of the other. In contrast, LG10068 (at 10^{-7} *M* or 10^{-8} *M*) alone had very little ability to inhibit development of these lesions, but a combination of LG10068 (at 10^{-8} *M*) and troglitazone (at 10^{-5} *M* or 10^{-6} *M*) almost completely inhibited (by 85% and 100%, respectively; both *P* values <.05) the development of mammary lesions. The expression of PPARγ and RXRα remained unchanged with the various treatments, whereas the expression of RARα was substantially reduced after treatment with the combination of retinoic acid and troglitazone.** ***Conclusions:*** **To our knowledge, this is the first report showing the possibility of a PPARγ ligand having chemopreventive activity. Furthermore, an RXR-selective retinoid, LG10068, appears to enhance this activity. [J Natl Cancer Inst 2000; 92:418–23]**

Breast cancer is one of the leading causes of cancer-related deaths. Clearly, the prevention of breast cancer is the best approach to this disease. For example, blockers of estrogen receptors (ERs), including tamoxifen and raloxifene, appear to diminish the frequency of breast cancer by about 50% in postmenopausal women *(1)*. We and other investigators *(2,3)* have shown that activation of the peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear hormone receptor superfamily, by thiazolidinediones including the synthetic ligand troglitazone inhibited proliferation of cultured breast cancer cells. Furthermore, all-*trans*-retinoic acid (hereafter referred to as retinoic acid), a ligand for another nuclear hormone receptor, i.e., retinoic acid receptor (RAR), enhanced the inhibition of proliferation of breast cancer cells. The combination of troglitazone and retinoic acid caused marked apoptotic cell death of tumors induced by MCF-7 breast cancer cells in immunodeficient mice without causing toxic effects in these animals *(3)*.

The PPARγ is a member assigned to the subfamily of nuclear hormone receptors that includes receptors for retinoic acid and thyroid hormone *(4)*. The PPARγ heterodimerizes with retinoid X receptor (RXR) and binds to DNA by recognizing target sequences that have a direct repeat of core recognition motifs (AGGTCA) spaced by one nucleotide. Activation of PPARγ results in expression of genes associated with many different aspects of differentiation, cellular development, and general physiology, including differentiation of adipocytes, lipid metabolism, and glucose homeostasis *(5,6)*. The natural PPARγ ligand appears to be 15-deoxy-$\Delta^{-12,14}$ prostaglandin J2, but a variety of polyunsaturated fatty acids including linoleic acid can also activate PPARγ *(7–9)*. Furthermore, nonsteroidal anti-inflammatory agents, such as indomethacin, can bind and activate PPARγ *(10)*. A series of thiazolidinediones, including troglitazone and pliglitazone, are useful for the treatment of type II adult-onset diabetes. Troglitazone has been used for the treatment of nearly one million individuals with diabetes. The mechanism by which this class of agents lowers blood glucose is unclear, although these agents may enhance differentiation of adipocytes associated with increased function of their glucose pumps.

Retinoids mediate their activity through the RAR and RXRs, both of which are expressed in breast cancer cells, although RARβ may not be expressed in all breast cancers *(11)*. The RAR and RXRs bind to specific retinoic acid-response elements and regulate the transcription of a variety of target genes in a ligand-dependent manner *(12,13)*. The all-*trans*-retinoic acid, an RAR-specific ligand, selectively inhibits the growth of human ER-positive breast cancer cells *(14–16)*. These cells appear to express higher levels of RARα than ER-negative cell lines *(16,17)*. Inhibition of growth of ER-positive human breast cancer cells by retinoids requires transactivation of retinoid-responsive genes *(18)*. Often the inhibition of growth of breast cancer cells by retinoids is reversible with the removal of the ligand *(19)*. These compounds also are effective in preventing mammary carcinogenesis in rodents *(20)*.

The murine mammary gland organ culture model system has been effectively used to evaluate the ability of potential

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See "Notes" following "References."

chemopreventive agents to prevent the development of preneoplastic lesions *(21–24)*. Mammary glands of BALB/c mice are placed in organ cultures containing a variety of growth-promoting hormones and are treated with the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) to induce preneoplastic lesions *(24,25)*. The mammary epithelial cells isolated from these lesions, when placed into syngeneic hosts, develop into adenocarcinoma *(26)*. With the use of this technique, more than 150 potential chemopreventive agents have been tested *(27)*. Effective chemopreventive agents, such as retinoids, selenium, oltipraz, limonene, and vitamin D_3 analogues, are able to inhibit the formation of these lesions. The assay is highly reproducible and provides a good association with the efficacy of an effective chemopreventive agent in the two-stage skin carcinogenesis *in vivo* assay and in prevention of chemically induced mammary tumors *in vivo (21,23,28)*. In this report, we have analyzed the efficacy of troglitazone, with or without a retinoid, in preventing the formation of DMBA-induced mammary lesions in a murine mammary gland organ culture model.

Materials and Methods

Reagents. Troglitazone was dissolved in a solution containing 50% dimethyl sulfoxide (DMSO) and 50% ethanol. The all-*trans*-retinoic acid (Sigma Chemical Co., St. Louis, MO) and LG10068 (Ligand Pharmaceuticals, Inc., San Diego, CA) were dissolved in 100% ethanol and added to organ cultures at a concentration of less than 0.1%. (Exact concentrations used are shown with the appropriate experiments.)

Protein extraction and western blot analysis. Murine mammary glands were homogenized in Triton X-100 containing lysis buffer. (The lysis buffer mixture contained 20 m*M* Tris buffer [pH 8.0], 137 m*M* NaCl, 10% glycerol, 1% Triton X-100, 2 m*M* EDTA, and protease inhibitors and was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN.) Protein lysate was separated on a 10%–20% gradient polyacrylamide Ready gel (BioRad Laboratories, Hercules, CA), and western blotting was performed on polyvinylidene fluoride membranes (Immobilon; Millipore Corp., Bedford, MA). The western blots were probed with an antibody raised against PPARγ-amino acids 2–20 (N-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA) used at a 1 : 2000 dilution and subsequently with an antibody against mouse actin (Oncogene Research Products, San Diego, CA) used at a 1 : 1000 dilution. The membranes were stripped and probed sequentially with RARα, RXRα, RXRβ, or RXRγ (Santa Cruz Biotechnology Inc.; 1 : 200 dilution). Results were visualized after reaction with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Life Science Inc., Arlington Heights, IL).

Induction of preneoplastic lesions in mammary glands and their prevention by ligands of nuclear hormone receptors. Young, virgin BALB/c female mice, 3–4 weeks of age, were obtained from Charles River Laboratories, Wilmington, MA. All of the animal studies were approved by the University of Illinois Animal Review Board and were performed in accordance with institutional guidelines. The entire culture procedure has been described in detail previously *(21–25)*. Briefly, the mice were pretreated for 9 days with 17β-estradiol (1 μg in 0.1 mL of saline/animal) and progesterone (1 mg in 0.1 mL of saline/animal). They were then killed by cervical dislocation, and the thoracic pair of mammary glands was removed, placed on silk rafts, and incubated for 10 days in serum-free Waymouth MB752 medium (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) containing the following growth-promoting hormones: insulin (5 μg/mL), prolactin (5 μg/mL), aldosterone (1 μg/mL), and hydrocortisone (1 μg/mL). The carcinogen DMBA at a dose of 2 μg/mL in DMSO was added to the medium on day 3 for a duration of 24 hours to induce mammary lesions. The DMBA-containing medium was removed, and the mammary glands were incubated for an additional 14 days with medium containing only insulin. This procedure allowed the normal glands to undergo structural regression in which all the normal alveolar structures were disintegrated. However, the alveolar lesions in the carcinogen-treated glands behave differently. They acquired altered hormone responsiveness and continued to grow. The nuclear hormone receptor ligand analogues were included in the medium during the first 10 days of the *in vitro* culture to determine if they lowered the incidence of formation of mammary lesions. Throughout the culture period, the glands were maintained at 37 °C in an environment of 95% O_2 and 5% CO_2. At the end of the culture period, the glands were fixed in formalin, stained in alum-carmine solution, and evaluated for the presence or absence of mammary lesions. All hormones and chemicals were purchased from Sigma Chemical Co.

Statistical analysis. The statistical significance was determined with the use of the GENCAT computer software program specifically written for the generalized chi-squared analysis of categorical data using weighted least squares *(29)*. The *P* values were further subjected to Bonferroni correction. All *P* values are two-sided.

Results

To examine the efficacy of troglitazone and/or a retinoid in preventing mammary lesions, we incubated 10–20 mammary glands per group (239 glands in total) from BALB/c mice with appropriate growth hormones and exposed them for 24 hours to DMBA on day 3 of culture. Fig. 1, A, shows the presence of mammary alveolar lesions in DMBA-treated glands. Mammary glands were cultured for 10 days with troglitazone with or without a retinoid. The incidence of mammary lesions was calculated for each group and was reported as a ratio of the number of mammary glands showing lesions compared with the total number of mammary glands at risk. The percent inhibition of formation of lesions for each treatment group was calculated by the comparison of the incidence of lesions between the control and the treatment groups. A dose-related decrease in the number of glands exhibiting lesions occurred in both the troglitazone-treated group and the retinoic acid-treated group

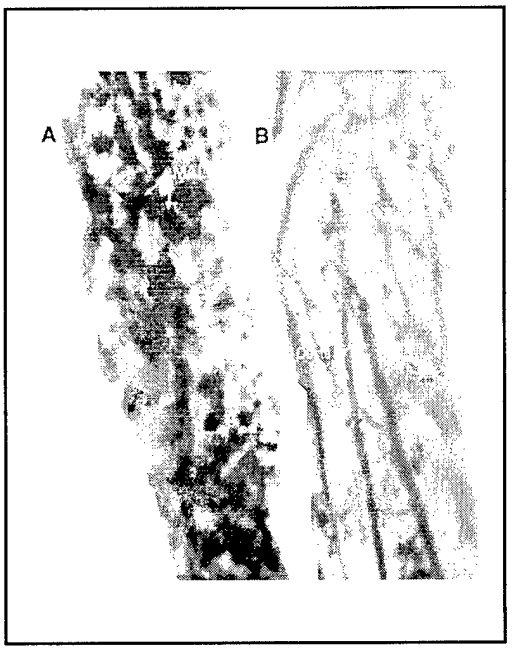

Fig. 1. Effects of nuclear hormone receptor ligands on the development of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary lesions. Mammary glands were incubated with insulin, prolactin, aldosterone, and hydrocortisone for 10 days either alone or with troglitazone and/or a retinoid. The glands were treated with DMBA on day 3, for 24 hours, during the initial 10 days of culture. The glands were further cultured for an additional 14 days with insulin alone. At that time, the glands were fixed in formalin and processed for morphologic evaluation. **Panel A:** mammary gland cultured with DMBA (24 hours) in the absence of chemopreventive agents. Representative mammary lesions (MAL) are shown. **Panel B:** representative photograph of the nearly normal ducts in a gland after culture with DMBA (24 hours) on day 3 of the culture plus troglitazone (10^{-5} *M*) and an RXR ligand (LG10068, 10^{-8} *M*) added to the culture from days 1 through 10 of culture (original magnification ×40).

(Table 1). Troglitazone at 10^{-6} *M* and 10^{-5} *M* inhibited mammary alveolar lesions by 31% and 60%, respectively. Glands treated with troglitazone (10^{-5} *M*) contained very few alveolar lesions, and no extensive dilation of ducts was evident. Toxicity in organ cultures is characterized by extensive dilation of mammary ducts, which results in disintegration of the gland structure *(24)*. Thus, troglitazone was not toxic at these concentrations.

The retinoic acid at 10^{-8} *M*, 10^{-7} *M*, and 10^{-6} *M* inhibited formation of lesions by 43%, 54%, and 77% (all *P* values <.05), respectively (Table 1). To evaluate the effects of the combination of troglitazone and retinoic acid, we used various concentrations of troglitazone (10^{-6} *M* to 10^{-5} *M*) and retinoic acid (10^{-8} *M* to 10^{-6} *M*). Inhibition of lesions with both ligands was similar to that with retinoic acid alone, with the exception of the combination of 10^{-5} *M* troglitazone and 10^{-7} *M* retinoic acid, which resulted in 100% inhibition of the lesions as compared with 66% by 10^{-5} *M* troglitazone or 54% inhibition mediated by 10^{-7} *M* retinoic acid. Since both troglitazone and retinoic acid were independently very active, the combined effect did not appear to be synergistic. There was no dilation of ducts or no noticeable toxicity observed with retinoic acid or with the combination of retinoic acid and troglitazone.

The PPARγ heterodimerizes with RXR, and each can simultaneously bind to its ligand, resulting in enhanced activity of this activated receptor complex. Thus, we examined the effect of troglitazone combined with an RXR ligand (LG10068). The RXR ligand (10^{-7} *M* to 10^{-8} *M*) was unable to inhibit DMBA-induced mammary lesions, and troglitazone (10^{-6} *M*) in this series of experiments inhibited mammary lesions by approximately 14% (Table 2). However, when the two were combined, the percent inhibition of development of alveolar lesions in mammary gland cultures was 85% or more (100%) showing that the two ligands together were clearly more effective than either alone (Table 2). The effect of the combination appears to be

Table 1. Effects of troglitazone and/or all-*trans*-retinoic acid on development of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced lesions in mouse mammary glands*

Group No.	Treatment	Concentration, *M*	Total No. of glands	Glands with lesions	Incidence, %	Inhibition,† %
1	None	None	30	26	87	N/A
2	Troglitazone	10^{-6}	20	12	60	31
3	Troglitazone	10^{-5}	20	7	35	60‡
4	Retinoic acid	10^{-8}	10	5	50	43
5	Retinoic acid	10^{-7}	10	5	50	54
6	Retinoic acid	10^{-6}	10	2	20	77‡
7	Troglitazone + retinoic acid	10^{-6} 10^{-8}	10	4	40	54
8	Troglitazone + retinoic acid	10^{-5} 10^{-8}	10	3	30	66‡
9	Troglitazone + retinoic acid	10^{-5} 10^{-7}	10	0	0	100‡
10	Troglitazone + retinoic acid	10^{-6} 10^{-6}	10	2	20	77‡
11	Troglitazone + retinoic acid	10^{-5} 10^{-6}	10	1	10	89‡

*All mouse mammary glands were treated with DMBA at a dose of 2 μg/mL for 24 hours on day 3. The chemopreventive agent(s) was present for the first 10 days of culture.

†% inhibition = [1 − (% incidence treatment group/% incidence control)] × 100. N/A = not applicable.

‡Statistically significant with the use of the GENCAT program specifically written for the generalized chi-squared analysis of categorical data using weighted least squares *(29)*. The two-sided *P* values were subjected to Bonferroni correction.

Table 2. Effects of troglitazone and/or the retinoid X receptor ligand LG10068 on development of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced lesions in mouse mammary glands*

Group No.	Treatment	Concentration, *M*	Treatment duration, days	Total No. of glands	Glands with lesions	Incidence, %	Inhibition,† %
1	None	None		10	7	70	N/A
2	Troglitazone	10^{-6}	0–10	10	6	60	14
3	Troglitazone	10^{-5}	0–10	10	1	10	85‡
4	LG10068	10^{-8}	0–10	10	8	80	0
5	LG10068	10^{-7}	0–10	9	6	67	5
6	Troglitazone + LG10068	10^{-6} 10^{-8}	0–10	10	1	10	85‡
7	Troglitazone + LG10068	10^{-5} 10^{-8}	0–10	10	0	0	100‡
8	Troglitazone	10^{-5}	0–4	10	2	20	71‡
9	Troglitazone	10^{-5}	4–10	10	2	20	71‡

*All mouse mammary glands were treated with DMBA at a dose of 2 μg/mL for 24 hours on day 3. For other details, *see* the "Materials and Methods" section.

†% inhibition = [1 − (% incidence treatment group/% incidence control)] × 100. N/A = not applicable.

‡Statistically significant (all *P*<.05), with the use of the GENCAT program specifically written for the generalized chi-squared analysis of categorical data using weighted least squares *(29)*. The two-sided *P* values were subjected to Bonferroni correction.

much enhanced compared with that of the individual compounds and may be synergistic. As shown in Fig. 1, A, DMBA induced mammary alveolar lesions in the absence of chemopreventive agents. However, the presence of the combination of troglitazone and LG10068, an RXR ligand, inhibited the development of DMBA-induced mammary lesions (Fig. 1, B).

In the same experiment, the effects of troglitazone on the initiation and promotion of lesions were investigated. When troglitazone (10^{-5} *M*) was present in the culture for only the first 4 days, we observed 71% inhibition of DMBA-induced mammary lesions. During the first 4 days of culture, DMBA was present for 24 hours on day 3 in the culture medium. Likewise, troglitazone, when present from days 4 through 10 of culture, was similarly able to inhibit development of mammary lesions (71% inhibition). During that period, DMBA was no longer present. Taken together, the results showed that troglitazone could inhibit both initiation and promotion of lesions of the mammary gland.

Expressions of RARα, RXRα, and PPARγ were examined in the control and experimental glands (Fig. 2). Results showed that RXRα and PPARγ were expressed in all glands; when normalized for the expression of actin, little change occurred in levels of RXRα or PPARγ with the various treatments. Mammary glands following the organ culture did not express RXRβ or RXRγ (data not shown). However, RARα was expressed in all glands. Results showed that there was a 25%–30% reduction in the RARα expression in the glands treated with retinoic acid (10^{-6} *M*) or troglitazone (10^{-6} *M*). The expression of RARα was further reduced by 60% when the glands were treated with a combination of troglitazone (10^{-6} *M*) and retinoic acid (10^{-6} *M*).

Discussion

Chemoprevention of cancer is clearly of great benefit to the individual and is less costly to society than is the treatment of established cancer. This one approach of cancer prevention is known as chemoprevention *(30)*. Compounds that can arrest either initiation or progression of breast carcinogenesis include ER antagonists, retinoids, monoterpenes, isoflavonoids, thiols, inhibitors of polyamine synthesis as well as prostaglandins, and vitamin D_3 analogues *(31–34)*. The synthetic ligand of PPARγ, troglitazone, has been shown to be effective against the proliferation of breast cancer cells; however, it has not been evaluated for its possible activity as a chemopreventive agent. In this study, troglitazone was able to inhibit the development of DMBA-induced mammary lesions, and this activity was potentiated by an RXR ligand, although by itself this ligand had no chemopreventive effects. On the other hand, unlike the RXR ligand, retinoic acid inhibited the development of mammary alveolar lesions in culture. The combination of retinoic acid and troglitazone resulted in additive chemopreventive activity. These experiments represent one of the initial steps toward the long-range goal of identifying effective chemopreventive agents for breast cancer. The lack of toxicity for most individuals receiving troglitazone for adult-onset diabetes, as well as the lack of adverse effects of several RXR ligands, including 9-*cis*-retinoic acid, makes the combination of troglitazone and an RXR analogue attractive for *in vivo* chemopreventive trials. Furthermore, several new thiazolidinediones are now available, and these compounds do not appear to have the idiosyncratic liver toxicity that occurs rarely with the administration of troglitazone.

How troglitazone, the synthetic ligand of PPARγ, inhibits transformation of mammary tissue is unclear. PPARγ is an important regulator of metabolism and storage of lipid *(35,36)*. This study and previous studies *(2,36,37)* showed that breast tissue expressed PPARγ. The amount of PPARγ expressed in normal breast epithelium appears to be less than that expressed in breast cancer *(3)*. Furthermore, the level of expression of PPARγ varies in normal mammary ducts, depending on whether the breasts are examined during the lactogenic period or the nonlactogenic period. We previously showed that, after culture with troglitazone and other natural and synthetic ligands of PPARγ, the MCF-7 breast cancer cells had an increased accumulation of fat and an increased expression of CD36 protein that was associated with active metabolism and storage of lipid *(3)*. However, these cancer epithelial cells did not cross-differentiate to adipocytes, as shown by their lack of expression of a number of key markers, such as aP2, lipoprotein lipase, and adipsin *(2,3)*. Our present data also showed that troglitazone had no marked effect on the level of expression of PPARγ, as shown by western blot analysis (Fig. 2). In addition, examination of these mammary cells by light microscopy revealed no increase in the level of apoptotic cell death, with the cells appearing perfectly normal. Studies have shown that PPARγ ligands can induce cell cycle arrest; the mechanism for this effect is unclear, but it may be a result of the receptor's reported involvement in inhibiting the activity of the E2F/DP family of transcriptional factors implicated in the initiation of the S phase of the cell cycle *(38)* and/or to antagonize the activity of the secondary signal proteins AP-1, STAT, and NF-κB *(39,40)*.

Thiazolidinediones can inhibit proliferation of a variety of cancer cells *(6,41,42)*. Activation of PPARγ promotes the differentiation and cessation of proliferation of human liposarcoma cells *(6)*. PPARγ ligands also induce differentiation and reverse the malignant phenotype of colon cancer cells *(41)*. Furthermore, PPARγ ligands can inhibit the proliferation of prostate cancer cells *in vitro* and in

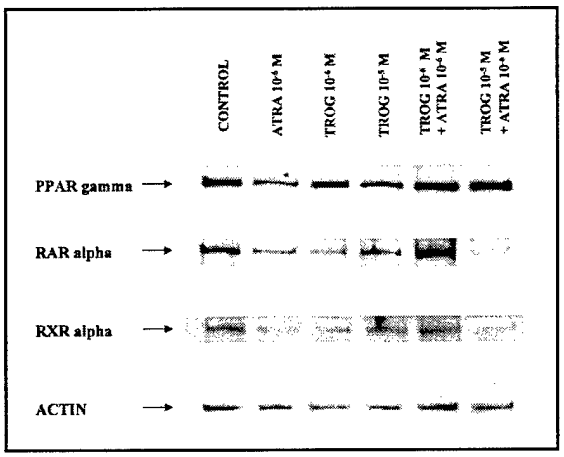

Fig. 2. Western blot, showing levels of expression of nuclear hormone receptors, e.g., peroxisome proliferator-activated receptor γ (PPARγ), retinoic acid receptor α (RARα), and retinoid X receptor α (RXRα), in mammary glands after different treatments: troglitazone (TROG; 10^{-5} *M* or 10^{-6} *M*), with or without all-*trans*-retinoic acid (ATRA; 10^{-6} *M*), as outlined in the text (*see* the "Materials and Methods" section and Table 1). Expression of actin controls is also shown.

laboratory animals *(42)*. Clinical studies in which troglitazone is given to individuals after radical prostatectomy for prostate cancer but who are still having a detectable serum prostate-specific antigen are now ongoing both in Los Angeles, CA, and Boston, MA. PPARγ ligands have also been shown to induce differentiation of myeloid leukemia cells *(43)*.

Previous studies by others and us *(32,33)* have shown that, after activation of the PPARγ receptor for several days, troglitazone could be removed and the breast cancer cell still had a markedly reduced capacity for clonogenic growth. Similarly, we showed here that exposure to troglitazone during either the first 4 days, i.e., initiation phase, or the final 6 days of the growth-promoting phase of culture suppressed transformation of the cells by DMBA (Table 2). In this experiment, the cells were pulse-exposed to the carcinogen only on day 3 of culture; therefore, the troglitazone appeared capable of inhibiting both the initiation and the promotion of cellular transformation.

The retinoic acid (RAR-specific ligand) also inhibited the carcinogen-induced development of mammary lesions. Previous studies have shown that the AP-1 transcriptional factor can be inhibited by retinoic acid *(44)* and may be responsible for the antitumor-promoting activity of retinoic acid *(45)*. Therapy with retinoic acid is strikingly successful for acute promyelocytic leukemia by inducing terminal differentiation of these leukemic cells *(46,47)*. Moreover, clinical trials have shown that *N*-4-hydroxyphenyl retinamide, a retinoid, may provide an effective therapy for some breast cancer patients *(32,33)*.

In this study, we found that combining a PPARγ ligand with a ligand specific for RXR (LG10068) enhanced the suppression of development of mammary lesions. A previous study *(48)* has shown that simultaneous activation of both receptors can result in synergistic activity in several assays of cultured cells as well as in augmented *in vivo* antidiabetic activity. Furthermore, we have previously shown that a PPARγ ligand and an RXR ligand can have enhanced antiproliferative effects against breast and prostate cancer cells *(3,42)*. Another study *(49)* has also shown that an RXR-specific agonist (LG10069) had chemopreventive activity against chemically induced rat mammary tumors; however, such activity for LG10068 has not been reported. Further studies are required to determine the target genes associated with this anticancer activity.

As far as we know, this is the first report showing the possibility of troglitazone, a PPARγ ligand, having chemopreventive activity. Troglitazone is a relatively nontoxic compound at a wide range of concentrations, but it is a potent inhibitor of the development of preneoplastic lesions of the mammary gland in organ culture. Also, an RXR- or an RAR-selective retinoid appears to enhance this chemopreventive activity; thus, the combination of a thiazolidedione and a retinoid, such as either retinoic acid or LG10068, may be a good candidate for an *in vivo* breast cancer chemoprevention study. Because of advances in the knowledge of genetics and epidemiology of breast cancer, individuals at high risk for developing breast cancer can be identified; these are the individuals who may receive the benefit from a chemoprevention regimen containing a PPARγ ligand combined with an appropriate RXR-selective retinoid.

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NOTES

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Novel therapeutic approach: organic arsenical (melarsoprol) alone or with *all-trans*-retinoic acid markedly inhibit growth of human breast and prostate cancer cells in vitro and in vivo

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Summary The organic arsenical known as melarsoprol (Mel-B) is used to treat African trypanosomiasis. Recently, another arsenical, As_2O_3 was shown to be effective in treatment of acute promyelocytic leukaemia. We have investigated the anti-tumour activities of Mel-B either with or without *all-trans*-retinoic acid (ATRA) using the MCF-7 human breast cancer cells, as well as the PC-3 and DU 145 human prostate cancer cells both in vitro and in vivo. The antiproliferative effects of Mel-B and/or ATRA against breast and prostate cancer were tested in vitro using clonogenic assays and in vivo in triple immunodeficient mice. Furthermore, the mechanism of action of these compounds was studied by examining the cell cycle, levels of bcl-2, apoptosis and antiproliferative potency using a pulse-exposure assay. Clonogenic assays showed that the cancer cell lines were sensitive to the inhibitory effect of Mel-B (effective dose that inhibited 50% clonal growth [ED_{50}]: 7×10^{-9} M for MCF-7, 2×10^{-7} M for PC-3, 3×10^{-7} M for DU145 cells. Remarkably, the combination of Mel-B and ATRA had an enhanced antiproliferative activity against all three cancer cell lines. Furthermore, the combination of Mel-B and ATRA induced a high level of apoptosis in all three cell lines. Treatment of PC-3 and MCF-7 tumours growing in triple immunodeficient mice with Mel-B and ATRA either alone or in combination markedly retarded tumour size and weight of the tumours without major side-effects. In conclusion, our results suggest that either Mel-B alone or with ATRA may be a useful, novel therapy for breast and prostate cancers. © 2000 Cancer Research Campaign

Keywords: melarsoprol; retinoid; breast cancer; prostate cancer; apoptosis

Breast and prostate cancers are the most common malignant diseases among women and men, respectively in Europe and the USA (Harris et al, 1997). Surgical resection or radiation therapy are potentially curative for localized diseases. Advanced breast and prostate cancers are associated with a poor prognosis, and conventional chemotherapies and radiation therapy are still of limited effectiveness. Endocrine therapies usually lead to either a partial or complete remission. However, subsequent relapse often occurs, and the disease re-emerges within a few years. Innovative approaches for advanced disease are necessary.

Arsenic is a naturally occurring element; pure arsenic is not common in the environment. Rather, it is usually found combined with one or more other elements such as oxygen, chlorine and sulphur. Arsenic combined with these elements is referred to as inorganic arsenic, whereas arsenic combined with carbon and hydrogen is referred to as organic arsenic. Maintaining a distinction between inorganic and organic arsenic is important, since the organic forms are usually less toxic. Traditional Chinese medicine has used an arsenic-containing remedy (Ai-Lin1) for many different ailments (Mervis, 1996). In the 1940s and 1950s, Fowler's solution which contained 1% potassium arsenite was frequently used for the treatment of chronic myelogenous leukaemia (Donofrio et al, 1987). Administration of inorganic arsenic, arsenic trioxide (As_2O_3), produced a high complete remission rate, as well as a relatively long-term survival in a significant proportion of individuals with acute promyelocytic leukaemia (APL) (Sun et al, 1992; Zhang et al, 1996; Chen et al, 1997; Soignet et al, 1998). Melarsoprol (Mel-B), an organic arsenical synthesized by complexing melarsen oxide with dimercaprol, has primarily been used for the treatment of African trypanosomiasis (Apted, 1970; Milord et al, 1992; van Nieuwenhove, 1992; Yunmbam et al, 1993). Mel-B induced apoptosis and inhibited in vitro growth of B-cell chronic lymphocytic leukaemia cell lines (König et al, 1997).

Retinoïds are natural and synthetic derivatives of vitamin A (Bollag et al, 1992). They prevent development as well as growth of several tumour types in animal models; and clinical trials have shown efficacy in individuals with APL, leukoplakia and recurrent squamous tumours of the head and neck (Gudas, 1992). Furthermore, retinoids inhibit the in vitro growth of a variety of cancer cells including those from leukaemias, breast, prostate and pancreas cancers (Douer et al, 1981; Pienta et al, 1993; Teelmann et al, 1993; Bollag et al, 1994; de Vos et al, 1996; Elstner et al, 1996). The regulation of cell growth and differentiation of normal, premalignant, and malignant cells by retinoids results from their effects on gene expression. These effects are mediated by nuclear retinoid receptors, which are ligand-activated transcription factors and members of the steroid hormone receptor superfamily (Evans, 1988; Mangelsdorf et al, 1994). Action of retinoids is mediated either by retinoic acid receptor-α (RAR-α), RAR-β, RAR-γ,

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...by retinoic X receptors (RXR) (Petkovich, 1992). *All-trans*-...noic acid (ATRA) is the first highly effective differentiation-...ing agent for remission induction in patients with APL ...ng et al. 1988; Warrell et al, 1993). It is the ligand for RARs.

To our knowledge, this is the first report of the effects of Mel-B ...the combination of Mel-B with ATRA for solid tumours. We ...that Mel-B or the combination of Mel-B and ATRA had ...ent anti-tumour activity against human breast and prostate ...cer cells both in vitro and in vivo.

MATERIALS AND METHODS

Mice

...enty male and twenty female 8-week-old BNX *nu/nu* mice ...re purchased from Harlan Sprague Dawley Inc, (Indianapolis, ...N, USA) and were maintained in pathogen-free conditions with ...diated chow.

Cell culture

...e human DU 145 and PC-3 prostate cancer cell lines and the ...CF-7 breast cancer cell line were obtained from American Type ...ulture Collection (Rockville, MD, USA) and were maintained in ...PMI-1640 medium (Gibco Laboratories, Grand Island, NY, ...SA) supplemented with 10% fetal calf serum (FCS; Gibco), 100 ... ml^{-1} penicillin and 100 mg ml^{-1} streptomycin.

Drugs

...elarsoprol {p-[(4,6,-diamino-s-triazin-2-yl)amino]dithiobenzene-...onous acid 3-hydroxypropylene ester} was a gift from the ...entral Disease Control (Atlanta, GA, USA), prepared in ...0 mg/5 ml ampoules and stored at 4°C. ATRA (Sigma, St Louis, ...O, USA) was dissolved in dimethyl sulphoxide (DMSO) at 10^{-2} ... for in vitro studies and 50 mg ml^{-1} for in vivo studies. It was ...ored at –80°C and protected from light.

Clonogenic assay in soft agar

...ency of drugs to inhibit the clonogenic growth of cancer cells ...nd the resulting ED50s (effective dose which inhibited 50% of ...onal growth) was determined by extensive dose–response ...udies in soft agar. The cells from 60–80% confluent liquid ...ultures were plated into 24-well flat bottom plates using a two-...yer, soft-agar system with a total volume of 400 µl, as described ...reviously (Munker et al, 1986). Drugs were added on day 0 prior ...o addition of feeder layer to the culture plates. After 14 days of ...ultivation, colonies (> 50 cells) were counted with an inverted ...icroscope. All experiments were done independently at least ...ree times in triplicate dishes per experimental point.

Western blot analysis of bcl-2

...CF-7, PC-3 and DU 145 cells were seeded at 1×10^5 and ...llowed to adhere overnight. The medium was replaced and to it ...as added either Mel-B (10^{-6} M) and/or ATRA (10^{-7} M). The cells ...ere incubated with these compounds for 72 h. Lysates were ...repared using Triton X-100 lysis buffer (20 mM Tris. Cl pH 8.0, ...137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, and 1 mM sodium orthovanadate). One hundred micrograms of the protein extract was added to each lane of a 10–20% gradient polyacrylamide gel. The membrane was blocked in phosphate-buffered saline (PBS)/5% non-fat milk (MLK) at room temperature and subsequently incubated with murine monoclonal anti-Bcl-2 (clone100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1 mg ml^{-1} in PBS/3% MLK for 2 h at room temperature, followed by incubation with horseradish peroxidase conjugated anti-mouse Ig (Amersham, Arlington Heights, IL, USA) at 1:1500 in PBS/3% MLK. An actin monoclonal antibody from Oncogene Research Products was used as a control protein. The results were visualized by enhanced chemiluminescence (Amersham), and densitometry was performed using the Alpha Imager 2000 digital imaging system with Alpha Ease version 3.0 software (Alpha Innotech Corp., San Leandro, CA, USA).

Cell cycle analyses

Cell cycle was analysed by flow cytometry. Briefly, cancer cells at <60% confluency were cultured either with or without analogues for 3 days in tissue culture medium, trypsinized, washed in DPBS, fixed in methanol and incubated for 30 min at 4°C in the dark with a solution of 5 mg ml^{-1} propidium iodide, 1 mg ml^{-1} RNAase (Sigma), and 0.1% Nonidet P-40 (Sigma). Analysis was performed immediately after staining using the CELLFit program (Becton Dickinson) whereby the S phase was calculated with a RFit model.

Measurement of apoptosis

After 5 days of cultivation of cancer cells with or without drugs (Mel-B, 10^{-6} M; ATRA, 10^{-7} M), the cells were trypsinized, washed with DPBS and analysed for apoptosis. Activation of an endonuclease results in extensive DNA cleavage and, thus, generates a large number of DNA strand breaks in apoptotic cells. DNA fragmentation was confirmed in our study by labelling of DNA strand breaks in apoptotic cells with BrdUTP (Li et al, 1995). This deoxynucleotide, once incorporated into the DNA strand breaks, is detected by a fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd antibody (Becton Dickinson, San Jose, CA, USA). Morphologically, cells undergoing apoptosis possess prominent features such as intense staining, highly condensed and/or fragmented nuclear chromatin, a general decrease in overall cell size, and cellular fragmentation into apoptotic bodies. These features make apoptotic cells relatively easy to distinguish from necrotic cells. For morphology, cytospin-slides with cultured cells were stained by Diff-Quick Stain Set. Apoptotic cells were enumerated in a total of about 300 cells by light microscopy.

Animal treatment protocol

Several animal studies were performed, using male BNX mice for the PC-3 prostate cancer experiments and female BNX mice for the MCF-7 breast cancer cells. Animals were bilaterally, subcutaneously injected with 5×10^6 of either PC-3 or MCF-7 cells per tumour in 100 µl Matrigel (Collaborative Biomedical Products, Bedford, MA, USA). Before injection of cells, the animals received 300 rads whole body irradiation. The mice were divided into four groups of five mice each, and received either diluant (DMSO) or experimental agents. Mel-B and ATRA (50 µl per injection) were administered intraperitoneally thrice weekly. One day after tumour

injections, mice were treated with either Mel-B alone, ATRA alone, or the combination of Mel-B and ATRA. Doses of Mel-B and ATRA were chosen from our preliminary studies in which various doses thrice weekly were given and mice were followed for toxicity. During the experiments, two mice died: one receiving Mel-B (10 mg kg^{-1}) and the other in the ATRA group (7.5 mg kg^{-1}) cohort. The cause of their deaths was unknown. Tumours were measured every week with vernier calipers. Tumour size was calculated by the formula: $a \times b \times c$, where a is the length and b is the width and c is the height in millimetres. At the end of the experiments, blood was collected from the orbital sinus for serum chemistries and haematopoietic analyses using Dupont Analyst Benchtop Chemistry System (Dade International, Newark, DE, USA) and by Serono-Baker 9000 Diff (Biochem Immuno-Systems, Allentown, PA, USA) respectively. Animals were sacrificed by carbon dioxide asphyxiation and tumour weights were measured after their careful resection.

Histology

Tumours and normal organs from sacrificed mice were fixed in 10% neutral buffered formalin and embedded in paraffin wax prior to histologic sectioning. Sections were stained with haematoxylin and eosin, and tumour necrosis and fibrosis were evaluated. Controls consisted of tumours and organs from mice not subjected to treatment.

Statistical analysis

All numerical data were expressed as the average of the values obtained, and standard deviation (s.d.) was calculated. For the in vitro studies, significance was determined by conducting a paired Student's *t*-test. For the in vivo studies, the statistical significance of the difference was analysed by the non-parametric Mann-Whitney *U*-test.

RESULTS

In vitro studies

Clonogenic assay

To study the effects of Mel-B, ATRA or their combination on clonogenic growth of cancer cells, the two-layer soft-agar system was performed. The MCF-7 breast cancer cells were very sensitive to the inhibitory effect of Mel-B in clonogenic assay (ED_{50}: 7×10^{-10} M); the prostate cancer cell lines, PC-3 and DU 145 had ED_{50}s of 2×10^{-7} M, and 3×10^{-7} M respectively (Figure 1). The ATRA alone at 10^{-7} M was only moderately inhibitory of the clonogenic growth of the PC-3 or DU 145 prostate cancer cells (30% and 27% respectively); however, the same concentration of ATRA (10^{-7} M) inhibited about 70% clonal growth of the MCF-7 breast cancer cells (Figure 2). Interestingly, the combination of both drugs (Mel-B, 10^{-7} M) and ARTA, 10^{-7} M) had at least an additive effect on clonal inhibition of each of the cancer cell lines (PC-3: 65% inhibition; DU 145: 74% inhibition; MCF-7: 90% inhibition) (Figure 2).

Apoptosis and bcl-2 levels

Exposure of the cells to the combination of Mel-B (2×10^{-6} M) and ATRA (10^{-7} M) for 5 days synergistically induced apoptosis in each of the cell lines [DU 145 (42%), PC-3 (51%) and MCF-7 cells (59%)], as measured by DNA fragmentation as compared to exposure of the cells to either agent alone (Figure 3). A similar, dramatic effect was observed when assessing apoptosis by morphology (data not shown).

The effect of Mel-B (10^{-6} M, 5 days), ATRA (10^{-7} M, 5 days) or

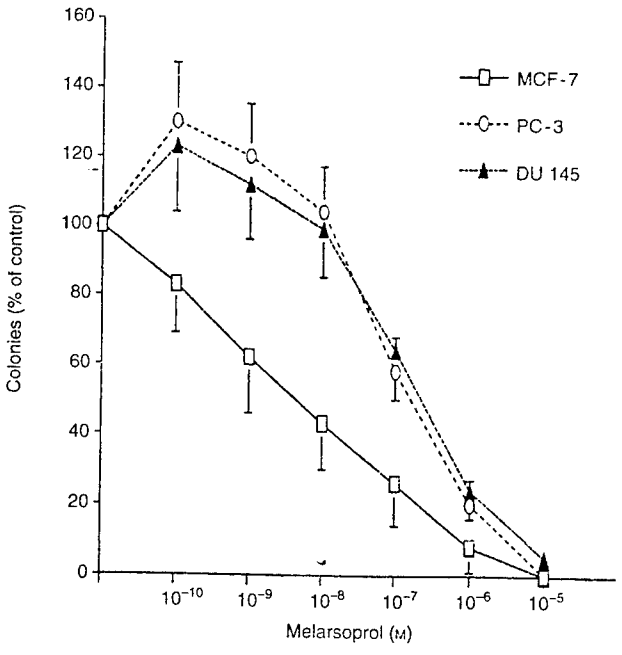

Figure 1 Dose–response studies of Mel-B: effect on clonal proliferation of breast and prostate cancer cells. Results are expressed as a mean per cent ± s.d. of control plates containing no drug. Each point represents a mean of at least three experiments with each experimental point having triplicate dishes. The drug was added to the culture dishes on day 0

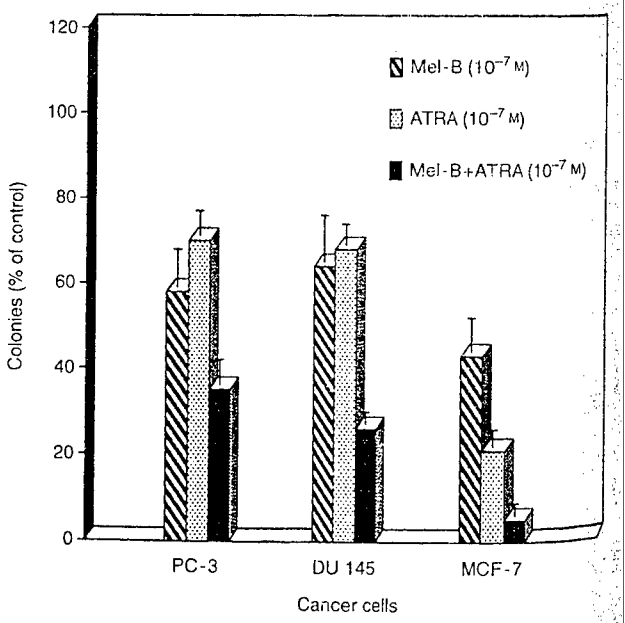

Figure 2 Effect of Mel-B and/or ATRA on clonal proliferation of breast and prostate cancer cells. Results are expressed as a mean percent ± s.d. of control plates containing no drug. Each point represents a mean of at least three experiments with each experimental point having triplicate dishes. The compounds were added to the culture dishes on day 0

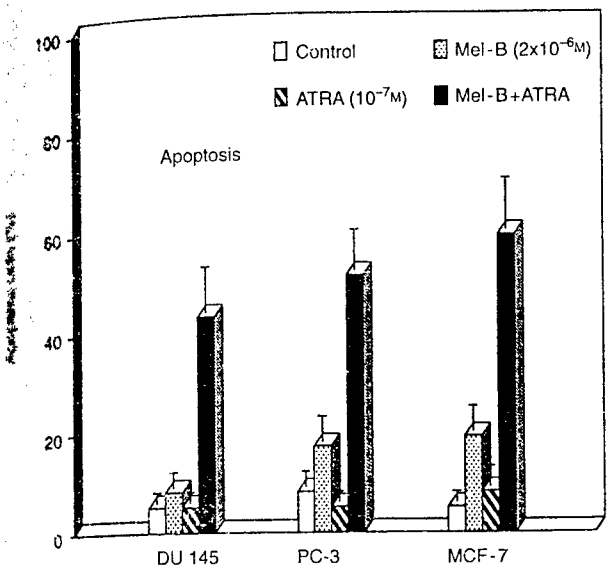

Figure 3 Apoptosis of cancer cells, as measured by labelling of DNA strand breaks with BrdUTR, after exposure of cells for 5 days to either Mel-B (2×10^{-6} M), ATRA (10^{-7} M) or both. Data expressed as per cent of apoptotic cells and represent the mean ± s.d. of two experiments. Controls are untreated cancer cells

both on the cellular content of bcl-2 protein was measured by Western blot (data not shown). When bcl-2 expression was corrected for actin expression, Mel-B decreased levels by 13%, ATRA lowered expression by 73%, and both together caused an 86% decrease in bcl-2 levels in the MCF-7 cells. Levels of bcl-2 were lower in the untreated PC-3 and DU 145 cells as compared to MCF-7, and these levels did not change markedly after exposure to Mel-B and/or ATRA (data not shown).

In vivo studies

We tested the ability of Mel-B and/or ATRA to inhibit the growth of MCF-7 breast and PC-3 prostate cancer cells growing in triple immunodeficient mice. Figure 4 shows the effect of Mel-B and/or ATRA on the size of PC-3 and MCF-7 tumours during 6 weeks of therapy. All of the treatment groups had statistically significantly smaller tumours than the diluant-control groups. Administration of Mel-B and ATRA alone remarkably suppressed the growth of the tumours. The size of the PC-3 tumours in mice treated with Mel-B at 5 and 10 mg kg^{-1} was similar, although the former was slightly smaller. The most potent effect was observed when Mel-B and ATRA were administered together to the mice bearing MCF-7 tumours. Besides determining the volume of the tumours over time, they were carefully dissected at the termination of the study and weighed. Results paralleled the volume measurements (data not shown). Tumour weights from each of the treated groups were statistically different from those of the control group, and the combination of Mel-B and ATRA was more potent than either alone. During the study, all mice were weighed once per week. The body weights of all treated groups were 91–101% of that of the control groups (data not shown). In general, all the mice of each of the cohorts looked healthy.

The blood chemistries (ten different studies including BUN, creatinine, liver enzymes and electrolytes) and haematopoietic parameters (including peripheral blood white and red cell counts, platelets

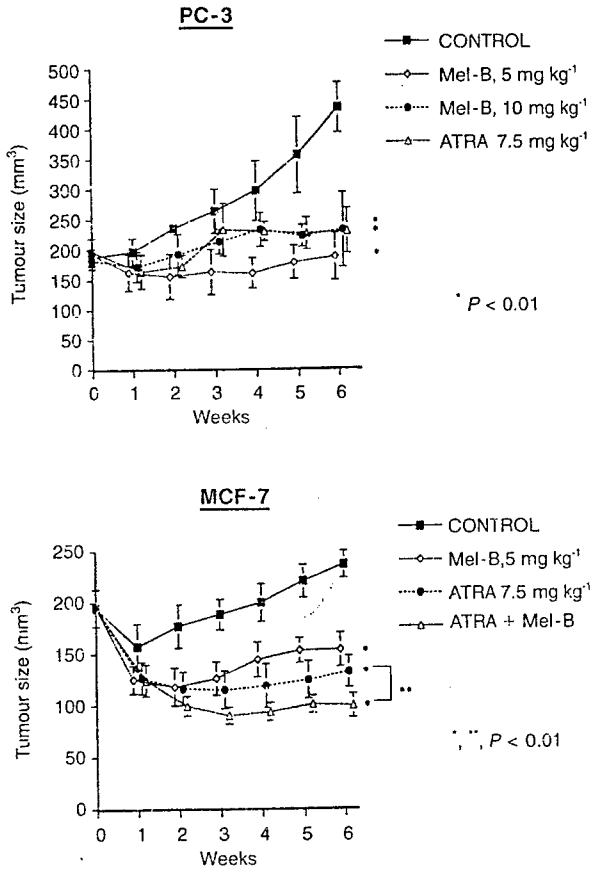

Figure 4 Volume of MCF-7 and PC-3 tumours in BNX mice receiving Mel-B and ATRA. Human MCF-7 breast or PC-3 prostate cancer cells (5×10^6) were injected subcutaneously, and Mel-B and ATRA were administered intraperitoneally (M, W, F), for 6 weeks to the BNX nude mice. Tumour volumes were calculated as the product of the length, width and height (see Materials and Methods section) of each tumour. Data are expressed as the mean ± s.d. for eight to ten tumours. Definitions: *, significantly different from control groups with $P < 0.01$ and **, significantly ($P < 0.01$) different between ATRA (7.5 mg kg^{-1}), and Mel-B and ATRA groups as determined by Mann–Whitney *U*-test

at the end of the study. The blood was collected from the orbital sinus while the animals were anaesthetized. No difference in the mean values of the blood chemistries were observed between the treated and untreated animals (data provided on request). The blood haematopoietic data showed little change between the cohorts, Group H (Mel-B + ATRA) had slightly higher white blood cell and platelet counts (data provided on request).

Histology

MCF-7

Tumours from control mice revealed poorly differentiated adenocarcinomas with about 30% necrosis and fibrosis (Figure 5A). Tumours from mice treated with Mel-B showed increased necrosis and apoptotic bodies (about 60% of the tumour mass) as well as fibrosis (Figure 5B). Tumours from mice receiving ATRA alone revealed similar changes in about 40% of each tumour section (data not shown). Tumours from mice treated with the combination of ATRA and Mel-B also contained a similar amount of tumour necrosis and apoptosis (Figure 5C).

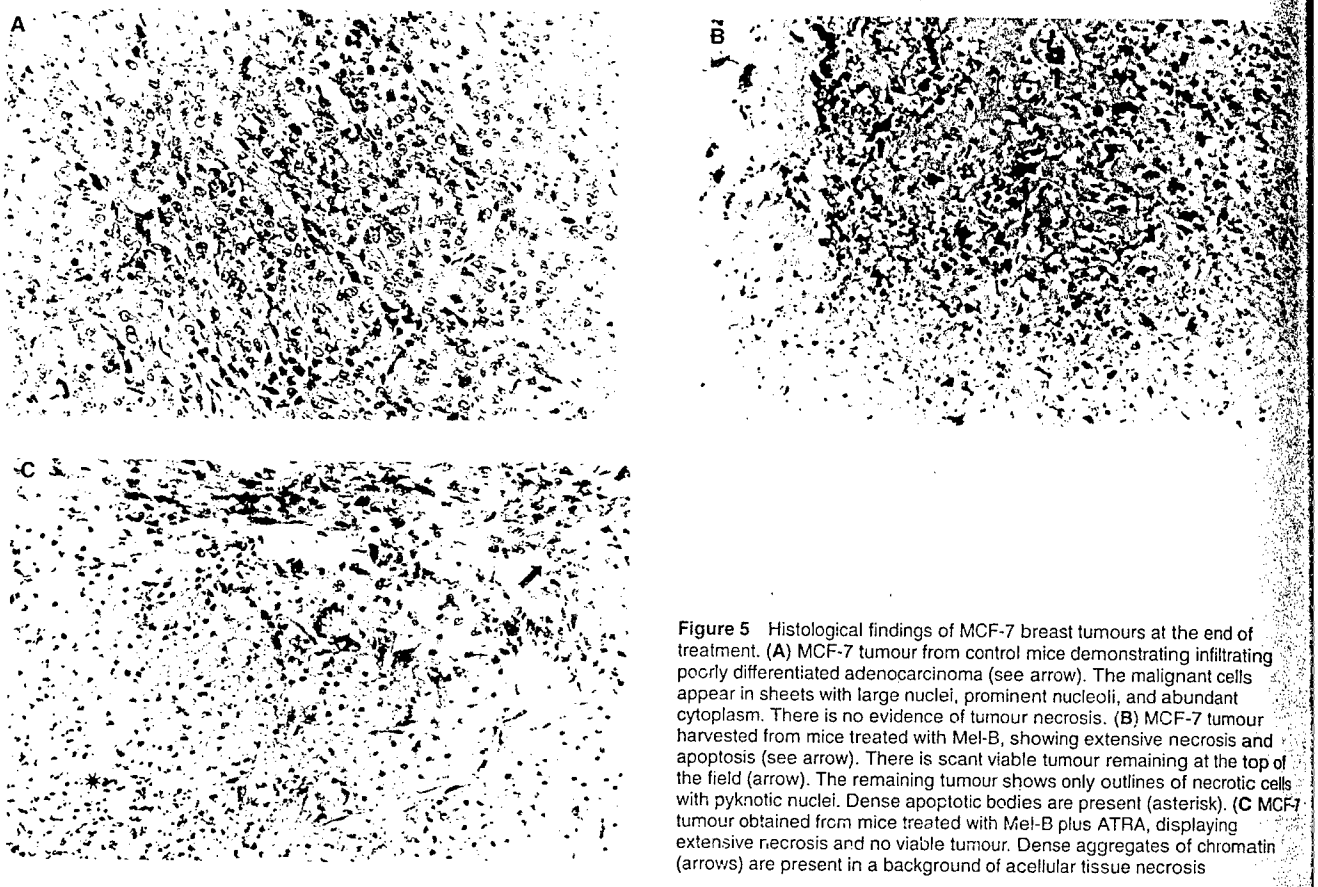

Figure 5 Histological findings of MCF-7 breast tumours at the end of treatment. (**A**) MCF-7 tumour from control mice demonstrating infiltrating poorly differentiated adenocarcinoma (see arrow). The malignant cells appear in sheets with large nuclei, prominent nucleoli, and abundant cytoplasm. There is no evidence of tumour necrosis. (**B**) MCF-7 tumour harvested from mice treated with Mel-B, showing extensive necrosis and apoptosis (see arrow). There is scant viable tumour remaining at the top of the field (arrow). The remaining tumour shows only outlines of necrotic cells with pyknotic nuclei. Dense apoptotic bodies are present (asterisk). (**C** MCF-7 tumour obtained from mice treated with Mel-B plus ATRA, displaying extensive necrosis and no viable tumour. Dense aggregates of chromatin (arrows) are present in a background of acellular tissue necrosis

PC-3

Controls revealed poorly differentiated carcinomas with small foci of necrosis and fibrosis which constituted approximately 20% of the area of the tumour section (data not shown). Tumours from mice treated with Mel-B at 5 or 10 mg kg^{-1} and/or ATRA 7.5 mg kg^{-1} revealed extensive necrosis and fibrosis [50–60% of each of the tumour sections revealed necrosis and histologic changes of apoptosis including formation of apoptotic bodies, and fibrosis involved approximately 30% of the tumour area (data not shown)].

DISCUSSION

Arsenic compounds have been generally considered to be a poison and a potent environmental carcinogen for human skin and lung cancers (Jaafar et al, 1993; Dong et al, 1994). Nevertheless, the inorganic arsenical, As_2O_3, induced apoptosis in the NB4 APL cell line associated with decreased expression of bcl-2 mRNA and protein (Chen et al, 1996, 1997). Furthermore, clinical studies in China and the USA have shown that As_2O_3 is an effective drug for patients with APL (Sun et al, 1992; Zhang et al, 1996; Chen et al, 1997; Soignet et al, 1998). Laboratory data suggest that the activity of arsenic in haematopoietic cell lines was independent of the expression of PML-RARα fusion product which is specific for the APL cells (König et al, 1997). The organic arsenical, Mel-B, is used for treatment of trypanosomiasis and this arsenical has been formulated for human use since 1949 (Friedheim, 1949). Recently, Mel-B was found to inhibit growth and decrease expression of bcl-2 in several chronic B-cell leukaemia cell lines (JVM-2, 183CLL, WSU-CLL) (König et al, 1997). Because arsenicals may have a broad range of activity, we examined the ability of Mel-B to inhibit the growth of human breast and prostate cancer cell lines.

Our clonogenic growth assays showed that MCF-7 breast cancer cells were very sensitive to the inhibitory activity of Mel-B. Furthermore, the strongest combined effects were observed in the cells treated with Mel-B and ATRA (Figures 1 and 2). The DU 145 prostate cancer cells are notably resistant to a variety of in vitro therapies (Thompson, 1994; Israel et al, 1995; deVos et al, 1996; Campbell et al, 1998); therefore, the sensitivity of these cells to clonal inhibition of proliferation by Mel-B and their prominent apoptosis with the combination of Mel-B and ATRA is notable. The PC-3 prostate cancer cells are moderately resistant to a variety of agents (Thompson, 1994; Israel et al, 1995; deVos et al, 1996; Campbell et al, 1998). These cells were also inhibited in their clonal growth by Mel-B. The Mel-B and ATRA at least additively decreased clonal growth of these prostate cancer cells. Comparison of the potency of inorganic As_2O_3 to Mel-B showed that Mel-B was about tenfold more potent in its antiproliferative activities than was As_2O_3 for each of the breast and prostate cancer cell lines, suggesting that Mel-B may be more active than As_2O_3 (data not shown).

The mechanism by which Mel-B mediates its anticancer activity is unclear. Prior investigation have reported that arsenicals affect protein tyrosine phosphorylation (Cavigelli et al, 1996; Chen et al, 1998). They can also decrease levels of glutathione which can result in DNA damage as a result of increased intracellular reactive oxygen molecules (Snow, 1992; Cavigelli et al, 1996).

Apoptosis or programmed cell death is of importance for the development and homeostasis of multicellular organisms (Fisher, 1994). Specific therapies have been designed to enhance the susceptibility of human cancers to undergo apoptosis (Yonish-Rouach et al, 1991; Symonds et al, 1994). Apoptosis is an active gene-directed cellular suicide mechanism; and many human genes contribute to its regulation, such as p53, c-myc and bcl-2 (Shi et al, 1992; Miyashita et al, 1993; Borsellino et al, 1995). We showed that the combination of Mel-B and ATRA dramatically and significantly increased the number of apoptotic cells in each of the three cancer cell lines, especially MCF-7 breast cancer cells (Figure 4). This effect was associated with a decrease in levels of bcl-2 protein in the MCF-7 cells (Figure 5). In contrast, levels of bcl-2 protein did not decrease in PC-3 and DU 145 prostate cancer cells after a similar treatment showing that decrease in existing levels of bcl-2 are not required for induction of apoptosis of these cells; but of note, bcl-2 levels were already relatively low in wild-type PC-3 and DU 145 cells. Soignet et al (1998) showed that apoptosis of APL cells by As_2O_3 was coincident with activation of caspases which are cysteine proteases important in mediating programmed cell death. Of interest after completion of our study, another investigative group noted that growth of As_2O_3-resistant NB4 APL cells was inhibited by the addition of ATRA to the cells (Gianni et al, 1998). Taken together, an arsenical and ATRA can have an enhanced antitumour effect in vitro; albeit, we do not understand the mechanism by which this occurs.

Our in vivo studies showed that the Mel-B as well as ATRA significantly inhibited the growth of PC-3 and MCF-7 cells. Furthermore, the treatment of MCF-7 breast cancer cells with the combination of Mel-B and ATRA was statistically superior to either Mel-B or ATRA alone. These data are consistent with our in vitro data. Blood chemistries and haematopoietic analyses showed that WBC and platelet numbers in the combination group were slightly higher than that of the control group, but all data were within the normal range (data available on request). Body weights in the experimental animals were within 10% of the control animals.

The histological data showed that all the MCF-7 breast and the PC-3 prostate tumours were poorly differentiated adenocarcinomas. Sections from mice treated with Mel-B and/or ATRA revealed extensive necrosis, apoptosis and fibrosis involving approximately 30–60% of the tumour area. Therefore, Mel-B had anticancer activities in vivo similar to what we observed in vitro. This activity occurred without major side-effects. The mechanisms of this anticancer effect remain unclear but are associated with prominent apoptosis. Toxicity was not discernible raising hopes that either Mel-B alone or when combined with ATRA may become a useful adjuvant therapy for breast and prostate cancers. This may be particularly true for the individuals who have minimal residual disease after curative attempt by surgery and/or radiotherapy.

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Synthesis and biological activities of the two C(23) epimers of 1α,23,25-trihydroxy-24-oxo-19-nor-vitamin D_3: Novel analogs of 1α,23(S),25-trihydroxy-24-oxo-vitamin D_3, a natural metabolite of 1α,25-dihydroxyvitamin D_3

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Running title: C(23) epimers of 1α,23,25$(OH)_3$-24-oxo-19-nor-vitamin D_3

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Abbreviations: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α,25$(OH)_2$-19-nor-D_3, 1α,25-dihydroxy-19-nor-vitamin D_3; 1α,23,25$(OH)_3$-24-oxo-19-nor-D_3, 1α,23,25-trihydroxy-24-oxo-19-nor-vitamin D_3; IR, infrared; ^{1}H NMR, proton nuclear magnetic resonance; ^{13}C NMR, carbon-13 nuclear magnetic resonance; PPh_3, triphenylphosphine; THF, tetrahydrofuran; DMSO, dimethylsulfoxide; CH_2Cl_2, methylene chloride; BOMCl, benzyloxymethyl chloride; BOM, benzyloxymethyl ether; TBDMS, *tert*-butyldimethylsilyl ether; TMSCl, trimethylsilyl chloride; PPTS, pyridinium *p*-toluenesulfonate; LDA, lithium diisopropylamide; TMS, trimethylsilyl ether; MeOH, methanol; MCPBA, meta chloroperbenzoic acid; DMF, dimethylformamide; Hunig's base, diisopropylethylamine; Pd/C, palladium on carbon; n-BuLi, n-butyl lithium.

Abstract

In a previous report, we indicated that 1α,23(S),25-trihydroxy-24-oxovitamin D_3 [1α,23(S),25$(OH)_3$-24-oxo-D_3], a natural metabolite of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] is almost equipotent to 1α,25$(OH)_2D_3$ in suppressing parathyroid hormone (PTH) secretion (Lee *et al*, 1997 Biochemistry 36, 9429-9437). Also, 1α,23(S),25$(OH)_3$-24-oxo-D_3 has been shown to possess only weak *in vivo* calcemic actions. Thus, vitamin D_3 analogs structurally related to 1α,23(S),25$(OH)_3$-24-oxo-D_3 may have therapeutic value. Furthermore, biological activity studies of various synthetic analogs of 1α,25$(OH)_2D_3$ showed that the removal of carbon-19 (C-19) reduces the calcemic activity of 1α,25$(OH)_2D_3$. Therefore, in an attempt to produce vitamin D_3 analogs with a better therapeutic index, we synthesized C(23) epimers of 1α,23,25$(OH)_3$-24-oxo-19-nor-vitamin D_3 [1α,23,25$(OH)_3$-24-oxo-19-nor-D_3]. The two epimers were compared to 1α,25$(OH)_2$-19-nor-D_3 and 1α,25$(OH)_2D_3$ in their ability to generate biological activities in several *in vitro* assay systems. In the assay measuring the suppression of parathyroid hormone (PTH) secretion in bovine parathyroid cells, 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 was as potent as 1α,25$(OH)_2$-19-nor-D_3 but was less potent than 1α,25$(OH)_2D_3$. In the same assay 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3 exhibited greater potency than 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3. In the assays measuring the ability of vitamin D compounds to inhibit clonal growth and to induce differentiation of human promyelocytic leukemia (HL-60) cells, 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 was less potent than 1α,25$(OH)_2$-19-nor-D_3 but was equipotent to 1α,25$(OH)_2D_3$. More importantly, in the same assays, 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3 was more potent than 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 and was equipotent to 1α,25$(OH)_2$-19-nor-D_3. Also, the vitamin D receptor-mediated transcriptional activity of 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3 was almost equal to that of 1α,25$(OH)_2$-19-nor-D_3, but higher than that of 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3. This finding explains in part the greater *in vitro* biological activities of 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3. In summary, our results indicate that 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3 and to a lesser extent 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3

are potent 19-nor vitamin D_3 analogs, which suppress PTH secretion in bovine parathyroid cells and strongly inhibit clonal growth and induce differentiation of HL-60 cells *in vitro*.

Key words: $1\alpha,25(OH)_2D_3$, $1\alpha,25(OH)_2$-19-nor-D_3, $1\alpha,23(R),25(OH)_3$-24-oxo-19-nor-D_3, $1\alpha,23(S),25(OH)_3$-24-oxo-19-nor-D_3, steroid.

INTRODUCTION

The role of the seco-steroid hormone, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] in the normal development of the skeleton and maintenance of calcium homeostasis is well established.[1] In addition to its role in mineral homeostasis, 1α,25$(OH)_2D_3$ has been shown to induce differentiation of myeloid leukemia cells.[2-7] Other biological activities include its role in immune suppression, treatment of the hyperproliferative skin disorders, and suppression of parathyroid hormone (PTH) secretion.[1,8] However, doses of 1α,25$(OH)_2D_3$ required to obtain therapeutically useful effects produced severe hypercalcemia.[9] This complication of hypercalcemia has limited the use of 1α,25$(OH)_2D_3$ as a therapeutic agent. Therefore, efforts have been directed towards synthesizing analogs of 1α,25$(OH)_2D_3$ that possess selectively noncalcemic actions such as differentiation of leukemic cells without producing hypercalcemia.[6,10,11] To date, hundreds of synthetic analogs of 1α,25$(OH)_2D_3$ have been synthesized and tested for their non-calcemic actions.[12] Biological activity studies of various synthetic analogs of 1α,25$(OH)_2D_3$ have shown that removal of carbon-19 (19-nor) reduces the potency of the calcemic actions of 1α,25$(OH)_2D_3$ while the potency of the non-calcemic actions of the hormone are retained or enhanced.[12-16]

In a recent report, we indicated that 1α,23(S),25-trihydroxy-24-oxovitamin D_3 [1α,23(S),25$(OH)_3$-24-oxo-D_3], a natural metabolite of 1α,25$(OH)_2D_3$ is almost equipotent to its parent in suppressing PTH secretion.[17] This finding has therapeutic significance as 1α,23(S),25$(OH)_3$-24-oxo-D_3 has been shown previously that it only generates minimal *in vivo* calcemic activity when compared to 1α,25$(OH)_2D_3$.[18] Therefore, in an attempt to reduce even further the minimal calcemic effects of 1α,23(S),25$(OH)_3$-24-oxo-D_3, we performed the present study (i) to synthesize the C(23) epimers of 1α,23,25$(OH)_3$-24-oxo-19-nor-D_3 and (ii) to compare these epimers to 1α,25$(OH)_2D_3$ and the analog 1α,25$(OH)_2$-19-nor-D_3 in their ability to generate the following biological activities: (a) to suppress PTH secretion in primary cultures of bovine parathyroid cells, (b) to inhibit clonal growth of human promyelocytic leukemia cells (HL-60 cells) (c) to induce differentiation of HL-60 cells [as assessed by the expression of CD11b surface protein (a differentiation marker)] and (d) to induce growth hormone production in rat

osteosarcoma cells (ROS 17/2.8) transfected with an osteocalcin VDRE/growth hormone gene contruct. The structures of $1\alpha,25(OH)_2D_3$, $1\alpha,23(S),25(OH)_3$-24-oxo-D_3, $1\alpha,25(OH)_2$-19-nor-D_3 and C(23) epimers of $1\alpha,23,25(OH)_3$-24-oxo-19-nor-D_3 are shown in Figure 1.

MATERIALS AND METHODS

General.

Melting points were determined using a Thomas-Hoover apparatus and are uncorrected. Infrared (IR) spectra were recorded with a Perkin-Elmer 1600 Series FTIR spectrophotometer. Optical rotations were measured with a Perkin-Elmer 241 polarimeter in a 1 dm cell. ^1H NMR spectra were recorded on either Bruker WM-250 MHz or Bruker AM-400 MHz spectrometers using TMS (0.0 ppm) as an internal standard. ^{13}C NMR spectra were recorded on a Bruker AM-400 MHz spectrometer at 100 MHz using $CDCl_3$ (77 ppm) as an internal standard. Mass spectra were obtained on routine intermediates with a Kratos MS 80RFA mass spectrometer under EI or CI conditions and for the final products on a Hewlett-Packard 5985B mass spectrometer. Thin layer chromatography (TLC) was performed on EM Science precoated silica gel 60F-254 glass-supported plates with 0.25 mm thickness. Spots were visualized by either ultraviolet light, exposure to iodine, or spraying with a 5% solution of phosphomolybdic acid (PMA) in ethanol. Flash chromatography was performed with ICN (70-220 mesh) of Kieselgel (230-400 mesh). Preparative thin layer chromatography was performed on Analtech Silica Gel GF plates (20 cm x 20 cm, 1000 μm thickness). Diethyl ether and THF were distilled from sodium/benzophenone ketyl under nitrogen. All the solvents and reagents used in the experiments were purified and dried according to the methods described by Perrin *et al.*[19] All reactions were run under an inert atmosphere of argon. High performance liquid chromatography (HPLC) was performed with a Waters Model 600E chromatograph equipped with a Waters photodiode array detector (Model 990) to monitor UV-absorbing material at 265 nm. Crystalline $1\alpha,25(OH)_2D_3$ was a gift from Dr. Milan R. Uskokovic of Hoffmann-La Roche, Nutley, New Jersey and

$1\alpha,25(OH)_2$-19-nor-D_3 was a gift from Dr. Hector F. DeLuca of University of Wisconsin, Madison, Wisconsin.

Chemical synthesis of C(23) epimeric mixture of $1\alpha,23,25(OH)_3$-24-oxo-19-nor-D_3 De-A,B-22-iodo-23,24-dinorcholestan-8-ol (6).

To a solution of Lythgoe's diol **5** (750 mg, 3.54 mmol) in benzene (50 mL), triphenylphosphine (930 mg, 3.54 mmol), imidazole (725 mg, 10.6 mmol) and iodine (897 mg, 3.54 mmol) were added at room temperature. The reaction became two phased with a white gel-like substance at the bottom of the flask. The mixture was stirred vigorously for 3 h. The reaction was quenched with water and extracted with benzene. The benzene layer was washed with 0.5N HCl (2 x 50 mL), 5% $NaSO_3$ and brine. Drying ($MgSO_4$) and concentration gave a thick oil which was chromatographed with 25% ethyl acetate/hexanes to afford the desired iodine **6** as a colorless oil (1.1g. 97%). $R_f = 0.65$ (25% ethyl acetate/hexanes).

^1H NMR (400 MHz., $CDCl_3$): δ 4.10 (br s, 1H), 3.33 (d, $J = 9.5$ Hz., 1H), 3.19 (m, 1H), 1.95-0.8 (m, 20H). MS m/e (rel int): 321(M^+, 30), 177 (76), 135 (28), 111(100), 95(42), 81(40), 67(25), 55(27).

De-A,B-22-benzenesulphonyl-23,24-dinorcholestan-8-ol (7a).

The iodide **6** (1.1 g, 3.4 mmol) was dissolved in anhydrous DMF (10 mL) under an argon atmosphere and sodium benzenesulfinic acid (1.12 g, 6.83 mmol) was added in one portion. The suspension eventually became a solution and was stirred overnight. The reaction was quenched with a saturated NH_4Cl solution and extracted with ether (2 x 50 mL). The ether layer was washed with water (2 x 25 mL) and brine. Drying ($MgSO_4$) and removal of the ether gave a thick oil which was chromatographed with 20% ethyl acetate/hexanes to give the desired sulfone **7a** as a colorless oil (1.07 g, 94%). $R_f = 0.14$ (25% ethyl acetate/hexanes).

^{1}H NMR (400 MHz., $CDCl_3$): δ 7.88 (d, *J* = 6.2 Hz., 2H), 7.63 (m, 1H), 7.54 (m, 2H), 4.02 (br s, 1H), 3.12 (d, *J* = 14.2 Hz, 1H), 2.82 (dd, *J* = 14.2, 9.6 Hz., 1H), 2.05 (m, 1H), 1.92 (d, *J* = 13.3 Hz., 1H), 1.17 (d, *J* = 8.8 Hz., 3H), 0.87 (s, 3H). IR (neat, cm^{-1}): 3530.3, 1296.2, 1143.4. ^{13}C NMR (100 MHz., $CDCl_3$): δ 140.25, 133.43, 129.16, 127.76, 68.89, 61.84, 55.74, 52.43, 42.02, 40.08, 33.43, 31.86, 26.93, 22.25, 19.87, 17.22, 13.21.

*De-A,B-22-benzenesulphonyl-23,24-dinorcholestan-8-benzyloxymethyl ether (**7b**).*

To a solution of the alcohol **7a** (230 mg, 0.68 mmol) in CH_2Cl_2 (5 mL), Hunig's base (0.24 mL, 1.4 mmol) and freshly distilled benzyl chloromethyl ether (0.14 mL, 0.9 mmol) was added at room temperature. The reaction was stirred overnight during which time it changed from a colorless solution to an orange solution. When all of the alcohol was reacted, as monitored by TLC, the reaction was quenched with water and extracted with ether (2 x 20 mL). The ether layer was washed with 0.1N HCl, brine, and dried ($MgSO_4$). Filtration and removal of ether gave a thick oil which was chromatographed with 10% ethyl acetate/hexanes to give the protected alcohol **7b** as a white solid (312 mg, 100%). R_f = 0.55 (25% ethyl acetate/hexanes).

^{1}H NMR (400 MHz., $CDCl_3$): δ 7.91 (m, 2H), 7.60 (m, 3H), 7.31 (m, 5H), 4.76 (d, *J* = 6.9 Hz., 1H), 4.66 (d, *J* = 6.9 Hz., 1H), 4.58 (s, 2H), 3.93 (br s, 1H), 3.17 (d, *J* = 14.2 Hz., 1H), 2.84 (dd, *J* = 14.2, 9.6 Hz., 1H), 1.19 (d, *J* = 6.6 Hz., 3H), 0.88 (s, 3H). IR-(neat, cm^{-1}): 1305.1, 1149.1. ^{13}C NMR (100 MHz., $CDCl_3$): 140.38, 138.03, 133.45, 129.20, 128.36, 127.84, 127.81, 127.57, 93.64, 74.59, 69.25, 61.94, 55.89, 52.32, 42.31, 40.29, 32.05, 30.29, 27.08, 22.56, 19.99, 17.78, 13.16. HRMS (CI, isobutane): Calculated for $C_{19}H_{27}O_3S$ (M - CH_2OCH_2Ph) 335.1681, found 335.1688. $[\alpha]^{20}_D$: +53.5 (c = 2.8, $CHCl_3$).

*De-A,B-22-benzenesulphonyl-24,25-diol-cholestan-8-benzyloxymethyl ether (**8a**).*

To a solution of the sulfone **7b** (500 mg, 1.1 mmol) in THF (5 mL), diisopropylamine (0.62 mL, 4.4 mmol) and tetramethylethylene diamine (0.5 mL) were added. After cooling the

reaction to -20°C, n-BuLi (2.5M in hexanes, 1.7 mL, 4.4 mmol) was added slowly in a dropwise manner over 10 min. The reaction turned yellow as the anion formed and was allowed to stir for 15 min. 2-methyl-3,4-epoxy-2-butan-2-ol (220 mg, 2.2 mmol) in THF (2mL) was added dropwise over 15 min. The reaction was stirred for 5 h and was quenched with a saturated NH_4Cl solution (10 mL) and extracted with ethyl acetate (3 x 20 mL). The organic layer was washed with brine (15 mL) and dried ($MgSO_4$). Filtration and removal of the ethyl acetate gave an oil which was chromatographed with 35% ethyl acetate/hexanes to give the recovered starting material (50 mg) and the desired product **8a** (500 mg, 81%). $R_f = 0.05$ (25% ethyl acetate/hexanes).

^{1}H NMR (250 MHz., $CDCl_3$): δ 7.90 (m, 2H), 7.62 (m, 3H), 7.34 (m, 5H), 4.69 (m, 3H), 4.56 (s, 2H), 3.89 (m, 1H), 3.52 (d, J = 9.2 Hz., 1H), 2.47 (d, J = 4.8 Hz., 1H), 0.66 (s, 3H). IR (neat cm^{-1}): 3476.2, 1284.7, 1141.4. HRMS (FAB): Calculated for $C_{32}H_{46}O_6S$ (M + H) 559.3093, found 559.3091.

De-A,B-24,25-dihydroxy-cholestan-8-benzyloxymethyl ether (8b).

To a solution of the sulfone **8a** (780 mg, 1.4 mmol) in methanol (20 mL) at 0°C, sodium phosphate dibasic (Na_2HPO_4) (5.95g, 42 mmol) and 5% sodium amalgam (9.4g, 2.1 mmol) were added. The mixture was stirred vigorously for 1h at 0°C. When the reaction was complete, as indicated by TLC, it was diluted with methanol and filtered through a pad of celite. Methanol was removed and the residue was diluted with brine (10 mL) and extracted with ethyl acetate (3 x 30 mL). Drying ($MgSO_4$), filtration and removal of ethyl acetate gave an oil which was chromatographed with 30% ethyl acetate/hexanes to give the pure diol **8b** (500 mg, 86%). R_f = 0.2 (25% ethyl acetate/hexanes).

^{1}H NMR (250 MHz., $CDCl_3$): δ 7.30 (m, 5H), 4.79 (d, J = 6.9 Hz., 1H), 4.68 (d, J = 6.9 Hz., 1H), 4.61 (s, 2H), 3.96 (m, 1H), 3.53 (m, 1H). IR (neat, cm^{-1}) 3425.2. ^{13}C NMR (100 MHz., $CDCl_3$): Major isomer: δ 138.17, 128.38, 127.89, 127.57, 93.71, 78.83, 74.96, 73.16, 69.22,

56.69, 52.42, 42.13, 40.63, 35.27, 32.71, 30.53, 28.11, 27.27, 26.59, 23.28, 22.80, 18.49, 17.98, 13.42. HRMS (FAB): Calculated for $C_{18}H_{33}O_2$ (M - OBOM) 281.2480, found 281.2481.

De-A,B-24-oxo-25-hydroxy-cholestan-8-benzyloxymethyl ether (9).

Oxalyl chloride (0.21 mL, 2.4 mmol) was dissolved in CH_2Cl_2 (10 mL) and cooled to -78°C. DMSO (0.31 mL, 4.8 mmol) in CH_2Cl_2 (0.5 mL) was added dropwise and the reaction mixture was stirred for 1h. The alcohol **8b** (500 mg, 1.2 mmol) in CH_2Cl_2 (10 mL) was added dropwise using a dropping funnel over an hour. A white precipitate was observed as the addition took place. After stirring at -78°C for 2h and at -40°C for 15 min, it was recooled to -78°C and triethylamine (1.2 mL, 8.4 mmol) was added dropwise. The reaction was allowed to warm up to -10°C on its own and stirred at this temperature until reaction was complete (about 5h). Water was added to the reaction and was extracted with CH_2Cl_2 (3 x 30 mL). Organic layer was washed with 0.1N HCl, brine, and dried ($MgSO_4$). Filtration and removal of CH_2Cl_2 gave a yellow oil which was chromatographed with 15% ethyl acetate/hexanes to give two products. The minor product (91 mg, 16%) corresponded to 24-oxo-25-thiomethoxy methyl ether. R_f = 0.8 (25% ethyl acetate/hexanes). The major product was the expected 24-oxo-25-hydroxy compound **9** (355 mg, 71%). R_f = 0.6 (25% ethyl acetate/hexanes).

^{1}H NMR (400 MHz., $CDCl_3$): δ 7.35 (m, 4H), 7.29 (m, 1H), 4.79 (d, J = 6.9 Hz., 1H), 4.68 (d, J = 6.9 Hz., 1H), 4.61 (m, 2H), 3.96 (m, 1H), 3.83 (s, OH), 2.51 (m, 2H). IR (neat, cm^{-1}): 3481.9, 1705.7. ^{13}C NMR (100 MHz., $CDCl_3$): δ 215.01, 138.14, 128.39, 127.88, 127.58, 93.69, 76.20, 74.85, 69.23, 56.45, 52.40, 42.14, 40.59, 35.04, 32.37, 30.49, 29.79, 27.23, 26.56, 22.78, 18.41, 17.96, 13.41. HRMS (EI): Calculated for $C_{18}H_{31}O_3$ (M - CH_2OCH_2Ph) 295.2273, found 295.2286. $[\alpha]^{20}_D$: +50.2 (c=1.4, $CHCl_3$).

*De-A,B-24-oxo-23,25-dihydroxy cholestan-8-benzyloxymethyl ether (**10**).*

A solution of LDA was prepared by adding n-BuLi (2.5M in hexanes, 0.28 mL, 0.72 mmol) to a solution of diisopropylamine (0.135 mL, 0.96 mmol) in THF (2 mL) at -20°C. After stirring for 40 min, a solution of the ketone **9** (50 mg, 0.12 mmol) in THF (2 mL) was added dropwise over 20 min. Trimethylsilyl chloride (0.12 mL. 0.96 mmol) was added after 5 min. The reaction was allowed to warm up to 0°C on its own and was stirred at this temperature for 3.5 h. At this point TLC showed disappearance of all the starting material and a new spot was observed with a very high R_f corresponsing to the silyl enol ether. A saturated $NaHCO_3$ solution was added to the reaction and was extracted with pentane (3 x 20 mL). The organic layer was washed with brine, dried ($MgSO_4$), filtered, and concentrated *in-vacuo*. The resulting oil was placed under vacuum overnight and was used in the next step without further purification. R_f = 0.75 (5% ethyl acetate/hexanes). The crude silyl enol ether was dissolved in CH_2Cl_2 (5 mL) and cooled to 0°C. *m*-Chloroperbenzoic acid (50%, 50 mg, 0.15 mmol) in CH_2Cl_2 (5 mL) was added very slowly using a dropping funnel over 30 min. The reaction was stirred at 0°C for 4h and then quenched with a saturated $NaHCO_3$ solution and extracted with CH_2Cl_2 (2 x 20 mL). The organic layer was washed with brine and concentrated *in-vacuo*. The resulting residue was dissolved in acetonitrile (3 mL) and treated with 0.5N HCl (7-10 drops). After stirring for 30 minutes, the reaction mixture was neutralized and acetonitrile was removed. The remaining residue was dissolved in ethyl acetate (50 mL) and was washed with brine (3 mL). Drying ($MgSO_4$), filtration, and concentration gave the crude oil which was chromatographed using 20% ethyl acetate/hexanes to give the desired dihydroxyketone **10** (40 mg, 78% for 2 steps). R_f = 0.35 (25% ethyl acetate/hexanes).

^{1}H NMR (400 MHz., $CDCl_3$): δ 7.34 (m, 5H), 4.79 (d, J = 6.9Hz., 1H), 4.67 (m, 2H), 4.60 (s, 2H), 3.96 (m, 1H), 2.92 (d, J = 8.0 Hz., OH- minor isomer), 2.89 (d, J = 7.9 Hz., OH-major isomer), 2.63 (s, OH-minor isomer), 2.57 (s, OH-major isomer). IR (neat, cm^{-1}): 3439.6, 1709.7. ^{13}C NMR (100 MHz., $CDCl_3$): major isomer: δ 217.08, 138.15, 128.39, 128.01, 128.68, 93.68, 77.20, 74.67, 71.03, 69.23, 57.09, 52.50, 42.40, 40.89, 40.64, 34.87, 32.52, 30.51, 27.72,

27.62, 27.42, 22.78, 17.96, 13.48. HRMS (FAB): Calculated for $C_{18}H_{31}O_3$ (M - OBOM) 295.2273, found 295.2261.

*De-A,B-24-oxo-23,25-(isopropylidenedioxy)-cholestan-8-benzyloxymethyl ether (**11a**).*

To a solution of the diol **10** (190 mg, 0.43 mmol) in anhydrous DMF (4 mL), 2,2-dimethoxypropane (2 mL) was added, followed by pyridinium *p*-toluenesulfonate (30 mg, 0.1 mmol). The reaction mixture was stirred for 2 days and worked up by diluting with water (30 mL) and extracting with ether (3 x 20 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated to give an oil which was chromatographed using 10% ethyl acetate/hexanes to give the ketal **11a** (160 mg, 78%). R_f = 0.86 (25% ethyl acetate/hexanes).

^{1}H NMR (400 MHz., $CDCl_3$): δ 7.33 (m, 5H), 4.79 (d, *J* = 6.9 Hz., 1H), 4.68 (d, *J* = 6.9 Hz., 1H), 4.61 (s, 2H), 4.31 (m, $1H_{23}$), 3.97 (m, 1 Hg), 1.52 (s, 3H), 1.48 (s, 3H), 1.44 (s, 3H), 1.29 (s, 3H). IR (neat cm^{-1}): 1737.0. ^{13}C NMR (100 MHz., $CDCl_3$): major isomer: δ 213.89, 138.13, 128.36, 127.87, 127.55, 100.15, 93.65, 79.39, 74.89, 72.02, 69.17, 57.04, 52.39, 42.26, 40.64, 36.27, 34.36, 31.49, 30.49, 29.39, 27.16, 26.24, 25.69, 25.07, 22.76, 17.95, 13.45. HRMS (EI): Calculated for $C_{28}H_{41}O_5$ (M - CH_3): 457.2954, found 457.2942.

*De-A,B-24-oxo-23,25-(isopropylidenedioxy)-cholestan-8-ol (**11b**).*

To a solution of de-A,B-24-oxo-23,25-(isopropylidenedioxy)-cholestan-8-(benzyloxy) methyl ether (75 mg, 0.16 mmol) in absolute ethanol (2 mL), 10% palladium on carbon (20 mg) was added. A balloon was filled with hydrogen gas and was connected to the reaction flask *via* an adapter. The reaction mixture was flushed with hydrogen gas several times. The balloon was filled again with hydrogen gas and the reaction mixture was stirred overnight under an atmospheric pressure of hydrogen. When the deprotection was complete, as monitored by TLC, the reaction mixture was filtered through a pad of celite. Ethanol was removed, and the residue was

chromatographed using 20% ethyl acetate/hexanes to give the alcohol **11b** (53 mg, 95%), R_f = 0.52 (25% ethyl acetate/hexanes).

^{1}H NMR (400 MHz., $CDCl_3$): δ 4.28 (dd, *J* = 10.5, 2.6 Hz., H_{23}-major), 4.25 (dd, *J* = 7.3, 4.8 Hz., H_{23}-minor), 4.05 (m, 1Hg), 1.49 (s, 3H-major), 1.48 (s, 3H-minor), 1.45 (s, 3H-major + minor), 1.42 (s, 3H-minor), 1.40 (s, 3H-major), 1.26 (s, 3H-major + minor), 0.97 (d, *J* = 6.6 Hz., 3H-minor), 0.94 (s, 3H-major), 0.92 (s, 3H-minor), 0.90 (d, *J* = 6.4 Hz., 3H major). IR (neat cm^{-1}): 3520, 1737.8. ^{13}C NMR (100 MHz., $CDCl_3$): major isomer: δ 213.86, 100.16, 79.39, 71.95, 69.28, 57.01, 52.59, 42.03, 40.46, 36.22, 33.57, 31.32, 29.38, 27.05, 26.23, 25.69, 24.48, 17.81, 17.42, 13.59. HRMS (EI): Calculated for $C_{20}H_{33}O_4$ (M - CH_3) 337.2379, found: 337.2374.

De-A,B-24-oxo-23,25-(isopropylidenedioxy)-cholestan-8-one (12).

Oxalyl chloride (0.025 mL, 0.284 mmol) was dissolved in CH_2Cl_2 (1 mL) and cooled to -78°C. DMSO (0.037 mL, 0.57 mmol) in CH_2Cl_2 (0.4 mL) was added dropwise and the reaction mixture was stirred for 45 min. The alcohol **11b** (50 mg, 0.142 mmol) in CH_2Cl_2 (1 mL) was added slowly in a dropwise manner over 15 min. The reaction mixture was allowed to warm up to -40°C on its own over 2.5 h and was kept at this temperature for 15 min. After the mixture was recooled to -78°C, triethylamine (0.16 mL, 1.14 mmol) was added dropwise. The reaction mixture was allowed to warm to -10°C on its own and was stirred at this temperature for 30 min. Water was added to the reaction mixture and was extracted with CH_2Cl_2 (2 x 20 mL). The organic layer was washed with brine, dried ($MgSO_4$), filtered and CH_2Cl_2 was removed to give an oil which was chromatographed with 25% ethyl acetate/hexanes to give the ketone **12** (47 mg, 96%). R_f = 0.54 (25% ethyl acetate/hexanes).

^{1}H NMR (400 MHz., $CDCl_3$): δ 4.29 (m, 1H-C_{23} major + minor), 2.45 (m, 1H), 2.26 (m, 2H), 1.04 (d, *J* = 6.6 Hz., 3H-C_{21}-minor), 0.97 (d, *J* = 6.1 Hz., 3H-C_{21}-major), 0.66 (s, 3H-C_{18}-

major), 0.64 (s, 3H- C_{18}-minor). IR (neat, cm^{-1}): 1737.4, 1714.5. ^{13}C NMR (100 MHz., $CDCl_3$): major isomer: δ 213.64, 211.82, 100.19, 79.40, 71.83, 61.92, 56.99, 49.95, 40.91, 38.99, 36.19, 31.53, 29.33, 27.35, 26.21, 25.67, 25.06, 23.98, 19.05, 17.96, 12.58. HRMS (CI, isobutane): Calculated for $C_{21}H_{35}O_4$ (M + H) 351.2535, found 351.2522.

*1-[(tert-butyldimethylsilyl)oxy]-23,25-(isopropylidenedioxy)-24-oxo-19-nor-vitamin D_3 tert-butyldimethylsilyl ether (**14**).*

To a solution of the phosphine oxide **13** (24 mg, 0.042 mmol) in dry THF (0.5 mL) at -78°C, n-BuLi (1.1M in hexanes, 0.05 mL, 0.055 mmol) was added dropwise. The reaction became dark orange to red in color from a colorless solution. After 5 min, the ketone **12** (10 mg, 0.028 mmol) in THF (0.3 mL) was slowly added dropwise. The red color disappeared gradually and became yellow by the end of addition. The reaction was stirred at -78°C for 1.5 h and then warmed to room temperature over 4 h. The solvent was removed and the residue was partitioned between ether and saturated $NaHCO_3$ solution. The organic layer was washed with brine, dried ($MgSO_4$) and concentrated to give an oil which was chromatographed on silica gel with 5% ethyl acetate/hexanes to give the desired coupled triene **14** (4.8 mg, 80%) and unreacted ketone **12** (7 mg). R_f = 0.9 (25% ethyl acetate/hexanes).

^1H NMR (400 MHz., $CDCl_3$): major isomer, δ 6.17 and 5.80 (d, J = 11.4 Hz., 2H-$C_{6,7}$), 4.32 (m, 1H-C_{23}), 4.08 (m, 2H-$C_{1,3}$), 2.82 (d, J = 11.5Hz., 1H-C_9), 0.96 (d, J = 6.2Hz., 3H-C_{21}), 0.87 (s, 9H, t-Bu), 0.86 (s, 9H, t-Bu), 0.57 (s, 3H-C_{18}), 0.07 (s, 3H, $SiMe_2$), 0.06 (s, 3H, $SiMe)_2$, 0.005 (s, 6H, $SiMe_2$). IR (neat cm^{-1}): 1738.7. ^{13}C NMR (100 MHz., $CDCl_3$): major isomer, δ 213.88 140.60, 133.77, 121.71, 116.25, 100.18, 79.42, 72.01, 68.09, 67.96, 56.93, 56.24, 45.98, 45.74, 43.70, 40.66, 36.80, 36.37, 32.14, 29.41, 28.66, 27.56, 26.25, 25.85, 25.83, 25.70, 25.07, 23.38, 22.21, 18.13, 18.09, 18.05, 12.13, -4.67, -4.76, -4.80, -4.93.

1α,23(R and S),25-trihydroxy-24-oxo-19-nor-vitamin D_3 (4).

To a solution of the protected 19-nor vitamin D_3 **14** (4.8 mg) in methanol (1 mL) and CH_2Cl_2 (0.1 mL), Dowex 50WX-400 resin (90 mg) was added. The mixture was stirred vigorously in the dark for 14 h. The reaction mixture was then filtered and concentrated *in-vacuo* to give a residue which was chromatographed with 100% ethyl acetate to give the pure 19-nor vitamin D_3 analog **4** (2.5 mg, 86%). $R_f = 0.16$ (75% ethyl acetate/hexanes).

^{1}H NMR (400 MHz., $CDCl_3$): δ 6.31 and 5.85 (d, J = 11.4 Hz., 2H-$C_{6\ \&\ 7}$), 4.71 (m, 1H-C_{23}-major), 4.64 (m, 1H-C_{23}-minor), 4.12 and 4.04 (m, 2H-$C_{1\ \&\ 3}$), 2.97 (d, J = 7.8 Hz., OH-C_{23} minor), 2.92 (d, J = 7.8 Hz., OH-C_{23}-major), 2.81 (dd, J = 12.3, 3.3 Hz., 1H-C_9), 2.74 (dd, J = 13.2, 3.7 Hz., 1H), 2.63 (s, OH_{25}-minor), 2.56 (s, OH_{25}-major), 2.48 (dd, J = 13.4, 3.3 Hz., 1H), 2.23 (m, 2H), 1.44 and 1.42 (s, 6H, $C_{26\ \&\ 27}$), 1.09 (d, J = 6.6 Hz., 3H-C_{21}-minor), 1.06 (d, J = 6.5 Hz., 3H-C_{21}-major), 0.59 (s, 3H-C_{18}-major), 0.56 (s, 3H-C_{18}-minor). IR (neat, cm^{-1}): 3398.1, 1718.7. ^{13}C NMR (100 MHz., $CDCl_3$): major isomer: δ 217.03, 142.76, 131.28, 123.78, 115.45, 77.21, 71.04, 67.42, 67.22, 56.90, 56.36, 45.98, 44.65, 42.19, 40.96, 40.48, 37.23, 33.13, 28.89, 27.77, 27.75, 27.64, 23.43, 22.28, 18.14, 12.15. minor isomer: δ 216.07, 142.85, 131.22, 123.82, 115.38, 73.29, 71.04, 67.42, 67.22, 56.94, 56.19, 45.89, 44.65, 42.19, 40.80, 40.37, 35.42, 33.13, 29.68, 27.89, 27.75, 27.64, 23.43, 22.32, 20.06, 11.96.

Analysis of 19-nor vitamin D_3 analog 4 by HPLC.

A total of 2.4 mg of the 19-nor vitamin D_3 analog **4** was subjected to HPLC under the chromatographic conditions described in the legend to Figure 4. We observed two UV absorbing peaks, a major (peak 1) and a minor (peak 2) peak (Figure 4). The elution volume of each UV absorbing peak was as follows: peak 1, 334-348 mL and peak 2, 350-370 mL. The HPLC effluent corresponding to each UV absorbing peak was collected separately and rechromatographed on the same HPLC run described above to obtain each individual isomer in the pure form.

PTH secretion in primary cultures of bovine parathyroid cells.

Cell culture. Bovine parathyroid glands, obtained from a local slaughterhouse and transported to the laboratory in cold PBS, were digested with collagenase as previously described[20] and seeded at a density of 80,000 cells/cm^2 in DMEM/Ham's F-12 (1:1) containing 4% heat-inactivated newborn calf serum, 15 mM HEPES, 100 IU/mL penicillin, 100 µg/mL streptomycin 5 µg/mL insulin, 2 mM glutamine, and 1% nonessential amino acids. After 24h, cells were placed in medium containing 0.1% bovine serum albumin and 5 µg/mL transferrin in place of the serum. Cells were grown to confluency (6 days) in this serum-free medium.

Analysis of PTH secretion. Parathyroid cell cultures were prepared as described above and grown for 4 days in serum-free medium. The cells were then treated for 3 days with the vitamin D_3 compounds shown in Figure 1 at concentrations ranging from 10^{-11} to 10^{-7} M with daily changes of the medium. Steady state PTH secretion was determined by washing the cells three times with Dulbeccco's PBS and then placing them in serum-free medium for 3h. The medium was collected, centrifuged at 4°C, and analyzed for PTH using CH9 antibody as described previously.[20] The cell monolayers were dissolved in 0.1N NaOH and assayed for protein by the method of Bradford[21] using a kit from Bio-Rad Laboratories (Richmond, CA). PTH secretion is expressed as picograms of PTH per milligram of cell protein. All statistical analysis was undertaken using the analysis of variance (ANOVA) and student's t-test.

Colony formation of HL-60 cells in soft agar.

The ability of all the vitamin D_3 compounds shown in Figure 1 to inhibit cell proliferation of HL-60 myeloid leukemia cells was determined by soft agar colony assay. In this experiment, the HL-60 cells were seeded in a two layer soft agar system as described earlier.[22] The lower layer contained 0.5% agar in which various concentrations [10^{-11} to 10^{-7} M] of the vitamin D compounds were mixed and the upper layer contained 0.3% agar in which the HL-60 cells ($5x10^3$ cells/plate) were mixed. The assay was performed in 24 well plates, the total volume in each well being 400

μL. The HL-60 cells were incubated with the test compounds for 10 days at 37°C in a humidified atmosphere containing 5% CO_2 in air. The colonies containing 40 or more cells were then counted under an inverted microscope. The experiment was repeated thrice using duplicate dishes for all experimental points. All statistical analysis was undertaken using the student's t-test.

Expression of CD11b in HL-60 cells.

The ability of all the vitamin D_3 compounds shown in Figure 1 to differentiate HL-60 cells was assessed by analysis of expression of CD11b protein, a differentiation marker. Mid-exponentially growing HL-60 cells ($2x10^5$/mL) were treated for 3 days with different concentrations (10^{-9} and 10^{-8}M) of the vitamin D compounds and then analyzed by fluorescence activated cell sorting (FACS) for expression of CD11b. The HL-60 cells were incubated with a saturating concentration of murine CD11b antibody (Carpenteria, CA) for 45 min on ice, followed by incubation with a goat antimouse FITC-conjugated secondary antibody. A FITC-conjugated isotype control was used. Immunofluorescence was analyzed with a Becton-Dickinson FACScan flow cytometer using LYSIS II software. The results are expressed as % CD11b positive cells. All statistical analysis was undertaken using the student's t-test.

Transcriptional assay in ROS 17/2.8 cells.

To test the vitamin D receptor-mediated transcriptional activities of the 19-nor- vitamin D_3 compounds, ROS 17/2.8 cells were plated in 35-mm dishes at a density of 3 x 10^5/dish in Dulbecco's modified essential medium (DMEM) and 10% FCS. Fourty eight hours later, the cells were transfected with plasmid (2 μg/dish) containing the vitamin D response element (VDRE) from the human osteocalcin gene (GGTGACTCACCGGGTGAACGGGGGCATT).[23] This response element was inserted upstream of the thymidine kinase promotor/growth hormone fusion gene. All transfections were performed by the DEAE dextran method,[24] and the cells were treated for 1 min with 10% dimethyl sulfoxide. Ligands (at the indicated concentrations) in serum-free medium were added for 1h immediately after transfection. The control samples were treated with

vehicle only. The cells were then washed twice in phosphate-buffered saline (PBS) and added to DMEM with 10% fetal calf serum (FCS). Medium samples for measurements of growth hormone were collected 24h after ligand treatment. Growth hormone production from the reporter gene was measured by a radioimmunoassay (RIA) as described by the manufacturer (Nichols Institute, San Juan Capistrano, CA, U.S.A).

RESULTS

Synthesis and separation of two C(23) epimers of 1α,23,25(OH)$_3$-24-oxo-19-nor-D$_3$ 4.

C(23) epimeric mixture of 1α,23,25(OH)$_3$-24-oxo-19-nor-D$_3$ was synthesized by coupling A-ring phosphine oxide **13** with CD ring ketone **12** using Lythgoe's Horner-Wittig method.[25] The 19-nor A-ring phosphine oxide **13** was prepared from quinic acid, as previously described by Perlman *et al.*[26]

The appropriate CD ring ketone **12** was synthesized from Lythgoe's diol **5** as shown in Figure 2. Selective iodination of the primary alcohol with triphenylphosphine and iodine gave **6** in 97% yield. Displacement of the iodide with sodium salt of benzene sulfinic acid in DMF resulted in **7a** with a yield of 94%. Initially, TBDMS group was used as the protecting group for the C-8 alcohol. However, the selective deprotection of the TBDMS group in later steps was proven to be unsuccessful under the standard conditions. The deprotection required the use of HF which removed all the protecting groups. As a result, benzyloxymethyl ether **7b** (BOM) was chosen as a suitable protecting group for the C-8 alcohol. The deprotection of the BOM group proceeded under normal hydrogenation conditions and it survived the sodium-almagam reduction step which was required in removing the sulfone group.

The 23,25-dihydroxy-24-oxo side chain was introduced to the CD ring sulphone using the method reported by Takayama.[27] Coupling of the sulphone **7b** with 2-methyl-3,4-epoxy-butan-2-ol[28] gave **8a** as shown in Figure 2. Reductive removal of the sulphone with sodium-almagam, followed by Swern oxidation of the C-24 alcohol gave the ketone **9**. The α-hydroxylation of the ketone **9** was accomplished by oxidizing the silyl enol ether with MCPBA to give 2:1 mixture of

C- 23 epimers **10**. The dihydroxy group at C-23 and C-25 was protected as a ketal and the BOM group was removed by hydrogenation to give the alcohol **11b**. The C-8 hydroxy group was oxidized to the ketone using Swern's oxidation[29] to give CD ring ketone **12**.

Coupling of A ring phosphine oxide **13** with the CD-ketone **12** (Figure 3) was successful using Lythgoe's conditions. Thus, phosphine oxide **13** was treated with n-BuLi at -78°C to form the anion, followed by addition of ketone **12** gave the coupled product. Deprotection of the ketal and TBDMS groups using Dowex acidic resin gave the desired 19-nor analog **4** in 69% yield for the two steps. The 19-nor analog **4** was isolated as a mixture of diastereomers, epimeric at C-23. The ratio of the two diastereomers was approximately 2:1 as determined by the ^{1}H NMR spectrum. The two diastereomers were separated using the HPLC system described in Figure 4 and the individual quantities of the two diastereomers obtained were about 1.6 mg, the major compound and 0.8 mg, the minor compound. Thus, we obtained each individual diastereomer in pure form to study their biological activities.

The streochemistry at C-23 was determined by comparing our ^{1}H NMR data with Takayama and co-workers.[27] We followed Takayama's synthesis in preparing the same side chain resulting in two C(23) epimers of 1α,23,25(OH)$_3$-24-oxo-19-nor-D$_3$. On the basis of Takayama's assignment of *R* to C(23) by x-ray diffraction analysis of the major isomer, we also assigned our major isomer the *R* configuration. Takayama reported the ^{1}H NMR chemical shift of the C-23 proton of the major isomer with the *R* configuration to be more downfield at 4.72 ppm compared to that of the minor isomer with the *S* configuration which resonates at 4.66 ppm. The ^{1}H NMR data of compound **4** shows two peaks for the C-23 proton at 4.70 and 4.64 with a ratio of 2:1, respectively. We assigned the major isomer which is more downfield with chemical shift of 4.70 ppm the *R* configuration and the minor isomer with chemical shift at 4.64 ppm the *S* configuration.

Effect of 1α,25(OH)$_2$D$_3$ and 19-nor vitamin D$_3$ compounds on PTH secretion in cultured bovine parathyroid cells.

The biological activities of 1α,23,25(OH)$_3$-24-oxo-19-nor-D$_3$ epimers in parathyroid cells were determined by comparing the potencies of these compounds to those of 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-19-nor-D$_3$ in suppressing PTH secretion. Parathyroid cells were incubated with various concentrations of 1α,23(S),25(OH)$_3$-24-oxo-19-nor-D$_3$, 1α,23(R),25(OH)$_3$-24-oxo-19-nor-D$_3$, 1α,25(OH)$_2$-19-nor-D$_3$ and 1α,25(OH)$_2$D$_3$ for 3 days. The steady state rate of PTH secretion was then measured during a 3h period. As shown in Figure 5, 1α,25(OH)$_2$D$_3$, 1α,25(OH)$_2$-19-nor-D$_3$ and both epimers of 1α,23,25(OH)$_3$-24-oxo-19-nor-D$_3$ decreased PTH secretion in a dose dependent manner. The two compounds, 1α,23(S),25(OH)$_3$-24-oxo-19-nor-D$_3$ and 1α,25(OH)$_2$-19-nor-D$_3$ are equipotent and less active than 1α,25(OH)$_2$D$_3$. For example, 1α,23(S),25(OH)$_3$-24-oxo-19-nor-D$_3$ and 1α,25(OH)$_2$-19-nor-D$_3$ significantly ($P < 0.05$) decreased PTH secretion at a concentration of 1 x 10^{-9} M while 1α,25(OH)$_2$D$_3$ significantly ($P < 0.05$) decreased PTH secretion at a concentration of 1 x 10^{-11} M. The compound, 1α,23(R),25(OH)$_3$-24-oxo-19-nor-D$_3$ [significantly ($P < 0.05$) decreased PTH secretion at a concentration of 1 x 10^{-10} M] was more active than 1α,23(S),25(OH)$_3$-24-oxo-19-nor-D$_3$ and 1α,25(OH)$_2$-19-nor-D$_3$ and less active than 1α,25(OH)$_2$D$_3$.

Effect of 1α,25(OH)$_2$D$_3$ and 19-nor vitamin D$_3$ compounds on clonal growth of HL-60 cells.

Figure 6 shows the dose-response effects of all the vitamin D$_3$ compounds shown in Figure 1 on clonal inhibition of HL-60 cell growth in soft agar. It can be seen from Figure 6 that all the vitamin D$_3$ compounds tested exerted their effects on HL-60 cell proliferation in a dose-dependent manner. However, 1α,25(OH)$_2$-19-nor-D$_3$ was significantly more active than 1α,25(OH)$_2$D$_3$ in inhibiting the clonal growth of HL-60 cells. For example at 1 x 10^{-9} M the 1α,25(OH)$_2$-19-nor-D$_3$ allowed only 32 ± 4% (mean ± SE) of colonies to grow compared to cells treated with 1α,25(OH)$_2$D$_3$ where 85 ± 2.5% of cells grew ($P < 0.05$). Similarly, of the two epimers, the 1α,23(R),25(OH)$_3$-24-oxo-19-nor-D$_3$ was significantly more potent than the 1α,23(S),25(OH)$_3$-

24-oxo-19-nor-D_3. For example, at the same dose of 1 x 10^{-9} M, the 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 allowed 84 ± 3.5% of colonies to grow. The 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3 significantly enhanced the potency ($P < 0.05$) allowing only 42 ± 2.5% of the colonies to grow.

Effect of 1α,25$(OH)_2D_3$ and 19-nor vitamin D_3 compounds on the expression of CD11b protein in HL-60 cells.

The HL-60 cells were cultured for 3 days with the vitamin D_3 compounds shown in Figure 1 (1 x 10^{-9} and 1 x 10^{-8} M) and the expression of CD11b protein was tested. Figure 7 shows the effects of 1α,25$(OH)_2D_3$ and 19-nor vitamin D_3 compounds on the expression of CD11b surface protein by HL-60 cells. At 1 x 10^{-9} M, 1α,25$(OH)_2$-19-nor-D_3 was significantly more active than 1α,25$(OH)_2D_3$ in inducing expression of CD11b protein on the cell surfaces of HL-60 cells ($P < 0.05$). Almost 50% of the HL-60 cells expressed CD11b protein when cultured in the presence of 1 x 10^{-9}M 1α,25$(OH)_2$-19-nor-D_3, while only 30% of the cells expressed CD11b protein when cultured in the presence of an equimolar concentration of 1α,25$(OH)_2D_3$. The compound, 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 induced almost similar expression of CD11b protein on the surfaces of HL-60 cells when compared to 1α,25$(OH)_2D_3$. The 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3 exhibited significantly higher biological activity when compared to 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 ($P < 0.005$) and was equipotent to 1α,25$(OH)_2$-19-nor-D_3 in inducing expression of CD11b protein on HL-60 cells.

Transcriptional activities of 1α,25$(OH)_2D_3$ and 19-nor-vitamin D_3 compounds in ROS 17/2.8 cells.

To determine vitamin D receptor (VDR)-mediated transactivational activities, we assessed the abilities of all the vitamin D_3 compounds shown in Figure 1 to induce growth hormone production in ROS 17/2.8 cells transfected with an osteocalcin VDRE/growth hormone gene contruct. Figure 8 shows that the VDR-mediated transcriptional activity through the osteocalcin VDRE was dose-dependent for 1α,25$(OH)_2D_3$ and the 19-nor vitamin D_3 compounds. Each of the

vitamin D_3 compounds had very little transcriptional activity at 0.01nM. Detectable transcriptional activity was observed at 0.1nM. Among the four vitamin D_3 compounds, 1α,25$(OH)_2D_3$ had the highest and 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 had the lowest transcriptional activities. The compound, 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3 when compared to 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 was more active transcriptionally even at 1nM. The transcriptional activity of 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3 was slightly higher or almost similar to 1α,25$(OH)_2$-19-nor-D_3 at all concentrations of the ligands. Thus, the higher transcriptional activity of 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3 when compared to 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 correlated well with the enhanced biological potency of this epimer in terms of (i) suppression of PTH hormone in bovine parathyroid cells; (ii) inhibition of clonal growth of HL-60 cells and; (iii) expression of CD11b protein on HL-60 cells.

DISCUSSION

In our present study, we synthesized the C(23) epimers of 1α,23,25$(OH)_3$-24-oxo-19-nor-D_3 and studied their *in vitro* biological activities in several assay systems, and these effects were compared with those of 1α,25$(OH)_2$-19-nor-D_3 and 1α,25$(OH)_2D_3$. We observed that even though 1α,25$(OH)_2$-19-nor-D_3 was slightly less active than 1α,25$(OH)_2D_3$ in reducing PTH secretion in primary cultures of bovine parathyroid cells, both 1α,25$(OH)_2$-19-nor-D_3 and 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 were almost equipotent in reducing PTH secretion. These biological activities of 19-nor vitamin D_3 compounds are similar to our earlier findings which indicated that 1α,23(S),25$(OH)_3$-24-oxo-D_3, a natural intermediary metabolite of 1α,25$(OH)_2D_3$, was almost equipotent to 1α,25$(OH)_2D_3$ in suppressing PTH secretion.[17] In our present study, we presented new information that the C-23 epimer with the hydroxyl group in "R" orientation [1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3], exhibited a higher biological activity than the epimer with the hydroxyl group in "S" orientation [1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3] in bovine parathyroid cells. In HL-60 cells, unlike in parathyroid cells, the 19-nor compounds exhibited increased biological activity. 1α,25$(OH)_2$-19-nor-D_3 was significantly more potent than

$1\alpha,25(OH)_2D_3$ at inhibiting the clonal growth and inducing differentiation of HL-60 cells. This finding suggests tissue specific differences in biological activities of 19-nor vitamin D_3 compounds. In HL-60 cells, unlike in bovine parathyroid cells, the activity of $1\alpha,23(S),25(OH)_3$-24-oxo-19-nor-D_3 when compared to $1\alpha,25(OH)_2$-19-nor-D_3 was reduced. However, it is of interest to note that in HL-60 cells also like in bovine parathyroid cells $1\alpha,23(R),25(OH)_3$-24-oxo-19-nor-D_3 exhibited higher biological activity when compared to that of $1\alpha,23(S),25(OH)_3$-24-oxo-19-nor-D_3, and was almost equipotent or slightly more active than $1\alpha,25(OH)_2$-19-nor-D_3.

We and other investigators have also demonstrated that 19-nor analogs are highly potent in suppresing the growth of breast, prostrate and leukemia cells.[13,15,30,31] The activities of $1\alpha,25(OH)_2D_3$ and its analogs in regulating cell growth and differentiation are primarily VDR mediated. The higher VDR-mediated transciptional activity of $1\alpha,23(R),25(OH)_3$-24-oxo-19-nor-D_3 when compared to that of $1\alpha,23(S),25(OH)_3$-24-oxo-19-nor-D_3 in inducing growth hormone production in ROS 17/2.8 cells transfected with an osteocalcin VDRE/growth hormone gene construct provides in part an important explanation for its greater *in vitro* activities of regulating cell growth and differentiation and suppressing PTH secretion. At present, several different factors appear to play a role in determining the biological effects of vitamin D analogs. Studies of Peleg and others[32,33] have shown that 20-epi analogs of $1\alpha,25(OH)_2D_3$ induce a different conformational change in the VDR which results in the formation of tighter heterodimers with the retinoid X receptor. This phenomenon leads to increased transactivation of target genes. Studies have not yet determined whether the 19-nor vitamin D_3 compounds produce a conformational change in the nuclear VDR different from that produced by $1\alpha,25(OH)_2D_3$. Biological activity of vitamin D_3 compounds can also be influenced by the rate of cellular uptake and intracellular metabolism. Proteins present in the culture medium can decrease the uptake of vitamin D compounds by cultured cells.[34] The serum vitamin D binding protein (DBP), which has high affinity for all natural vitamin D metabolites, can reduce the biological activity of $1\alpha,25(OH)_2D_3$ and its analogs by retarding their cellular uptake.[31,34] Recently, Addo and Ray[35] reported that $25OHD_3$ analogs with modifications at C-19 position possessed almost equal binding affinity for

the DBP when compared to $25OHD_3$ suggesting that C-19 modification of $25OHD_3$ does not reduce significantly its binding to DBP. We tested the biological activities of the 19-nor vitamin D_3 compounds in the absence of FCS in the culture medium to minimize the differences in cellular uptake that could influence the biological activity. Another key factor in determining the biological activity of vitamin D compounds is their rate of metabolism. At present, the rates of metabolism of the 19-nor vitamin D_3 compounds have not been examined.

In summary, our results indicate that 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3 and to a lesser extent 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 are potent 19-nor vitamin D_3 analogs, which strongly inhibit clonal growth and induce differentiation of HL-60 cells and suppress PTH secretion in parathyroid cells *in vitro*, and thus offer an appealing therapeutic prospect. Our recent preliminary studies indicated that 1α,25$(OH)_2$-19-nor-D_3 was metabolized *via* the C-24 oxidation pathway in the perfused rat kidney, and we identified 1α,23,25$(OH)_3$-24-oxo-19-nor-D_3 as a major metabolite of 1α,25$(OH)_2$-19-nor-D_3. However, the stereochemistry of this metabolite has not been determined.[36] Based on our previous work[17] which established 1α,23(S),25$(OH)_3$-24-oxo-D_3 as the natural metabolite of 1α,25$(OH)_2D_3$, 1α,23(S),25$(OH)_3$-24-oxo-19-nor-D_3 is most likely the natural epimer and 1α,23(R),25$(OH)_3$-24-oxo-19-nor-D_3 is the unnatural epimer.

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FIGURE LEGENDS

Figure 1: Structures of 1α,25(OH)$_2$D$_3$, 1α,25(OH)$_2$-19-nor-D$_3$, 1α,23,25(OH)$_3$-24-oxo-D$_3$ and the two C(23) epimers of 1α,23,25(OH)$_3$-24-oxo-19-nor-D$_3$.

Figure 2: Scheme for the synthesis of De-A,B-24-oxo-23,25-(isopropylidenedioxy)-cholestan- 8-one (**11**).

Figure 3: Scheme for the synthesis of 1α,23,25(OH)$_3$-24-oxo-19-nor-D$_3$ (**4**) by coupling A-ring phosphine oxide **12** with CD ring ketone **11**.

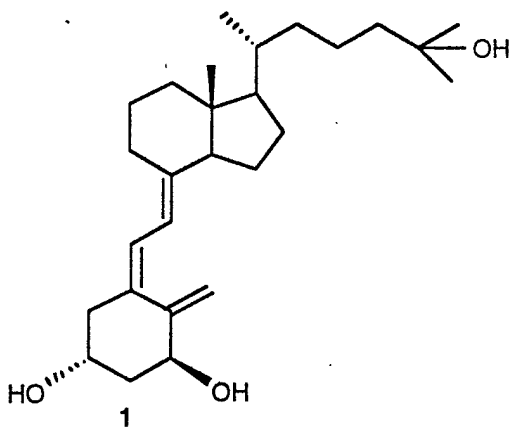
Figure 4: HPLC separation of the two C(23) epimers of synthetic 1α,23,25(OH)$_3$-24-oxo-19-nor-D$_3$: HPLC was performed on Zorbax-Sil column (9.4 mm x 25 cm) which eluted with hexane:2-propanol (95:5 v/v) at a flow rate of 2 mL/min. The UV absorption spectra of 1α,23(R),25(OH)$_3$-24-oxo-19-nor-D$_3$ (1) and 1α,23(S),25(OH)$_3$-24-oxo-19-nor-D$_3$ (2) are shown in the insert.

Figure 5: Suppression of PTH secretion by 1α,25(OH)$_2$D$_3$ and 19-nor vitamin D$_3$ compounds: Confluent cultures of bovine parathyroid cells were incubated with the specified concentration of 1α,25(OH)$_2$D$_3$, 1α,25(OH)$_2$-19-nor-D$_3$, 1α,23(R),25(OH)$_3$-24-oxo-19-nor-D$_3$ or 1α,23(S),25(OH)$_3$-24-oxo-19-nor-D$_3$ for 72 h. The media was changed every 24h and fresh concentration of the test analog was added each day. The cells were then washed, and steady state PTH secretion was determined during a 3h incubation. PTH was assessed by radioimmunoassay, and data were corrected for the total cell protein. The data are expressed as mean ± SD and represent combined data from two separate experiments. Values with the same character are significantly different from the respective control ($P < 0.05$).

Figure 6: Effects of various doses of $1\alpha,25(OH)_2D_3$ and 19-nor vitamin D_3 compounds on the clonal growth of HL-60 cells. The HL-60 cells were cultured with graded concentrations of either $1\alpha,25(OH)_2D_3$, $1\alpha,25(OH)_2$-19-nor-D_3, $1\alpha,23(R),25(OH)_3$-24-oxo-19-nor-D_3 or $1\alpha,23(S),25(OH)_3$-24-oxo-19-nor-D_3 [10^{-7} to 10^{-11}M] for 10 days and colonies were counted using an inverted microscope. The results are expressed as % of control plates which do not contain the various vitamin D_3 compounds. Each value represents the mean ± SE of three experiments with duplicate dishes.

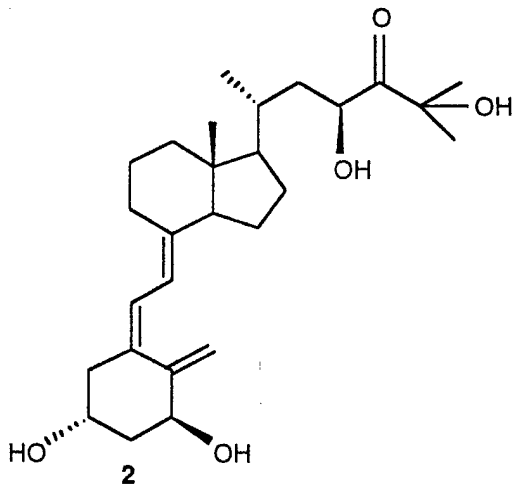
Figure 7: Effects of $1\alpha,25(OH)_2D_3$ and 19-nor vitamin D_3 compounds on the expression of CD11b antigens on HL-60 cells. The HL-60 cells were treated for 3 days with $1\alpha,25(OH)_2D_3$, $1\alpha,25(OH)_2$-19-nor-D_3, or the C(23) epimers of $1\alpha,23,25(OH)_3$-24-oxo-19-nor-D_3 [10^{-8} and 10^{-9}M] and then analyzed by FACS for the expression of CD11b. Each value represents the mean ± SE of three experiments with duplicate dishes.

Figure 8: Transcriptional activity of the 19-nor vitamin D_3 compounds in ROS 17/2.8 cells. The cells were transfected by the DEAE-dextran method with the fusion gene containing the osteocalcin VDRE attached to the thymidine kinase/growth hormone reporter gene. Immediately after transfection, the cells were treated with either $1\alpha,25(OH)_2D_3$ or 19-nor vitamin D_3 compounds for 24h in media containing 10% FCS. Culture medium was assayed for growth hormone production by radioimmunoassay (Nichols Institute). Each point of the dose-response curve is the mean of duplicate transfections and the results shown are representative of four transfection experiments.



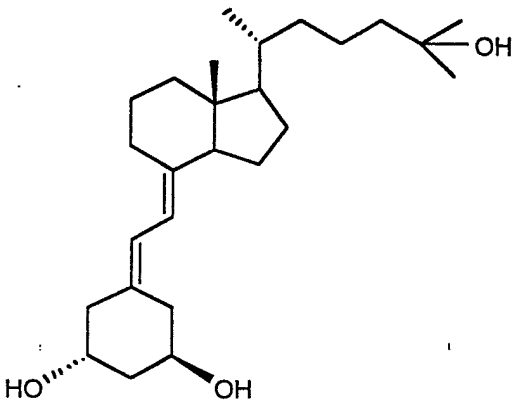
1

1α,25(OH)$_2$D$_3$



2

1α,23(S),25(OH)$_3$-24-oxo-D$_3$



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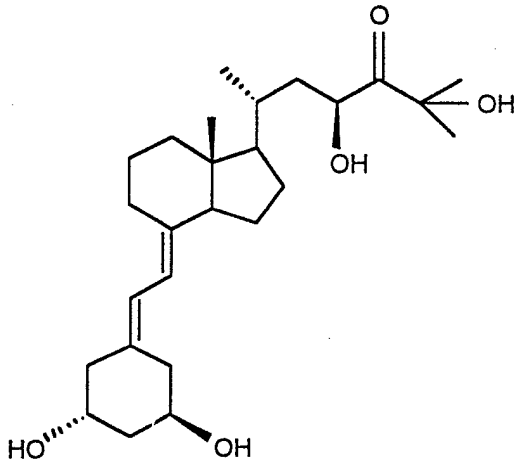
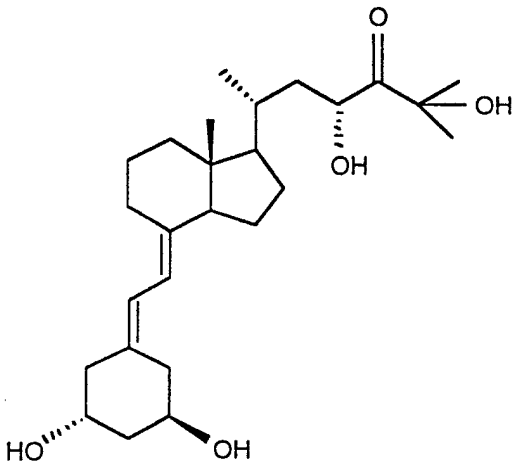


4a

1α,23(S),25(OH)$_3$-24-oxo-19-nor-D$_3$



4b

1α,23(R),25(OH)$_3$-24-oxo-19-nor-D$_3$

Fig. 1

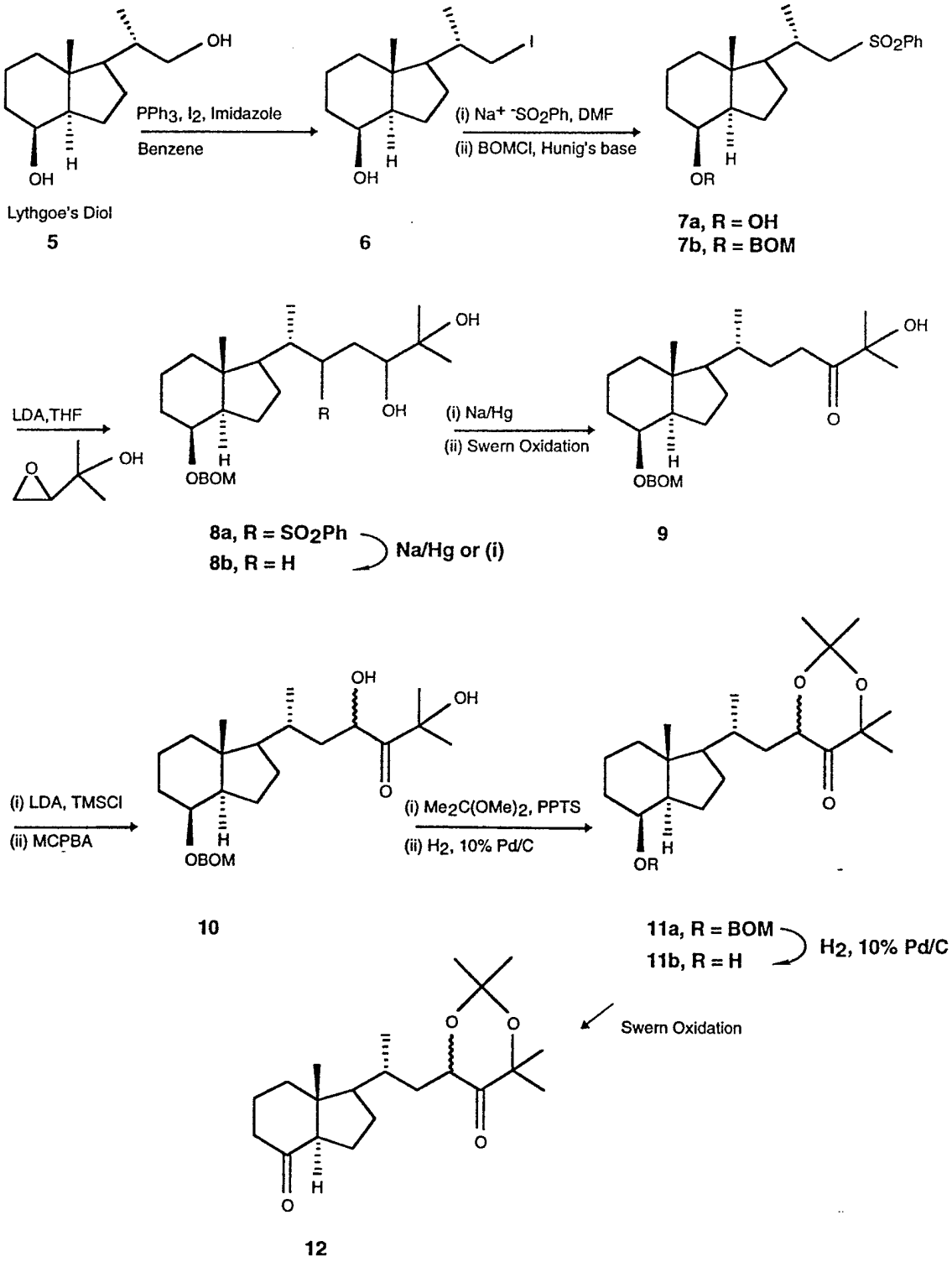

OH
OH
H
PPh3, I2, Imidazole
Benzene
I
OH
H
(i) Na+ -SO2Ph, DMF
(ii) BOMCl, Hunig's base
SO2Ph
OR
H
Lythgoe's Diol
5
6
7a, R = OH
7b, R = BOM
LDA,THF
O
OH
OH
R
OH
H
OBOM
(i) Na/Hg
(ii) Swern Oxidation
OH
O
H
OBOM
8a, R = SO2Ph
8b, R = H
Na/Hg or (i)
9
(i) LDA, TMSCl
(ii) MCPBA
OH
OH
O
H
OBOM
(i) Me2C(OMe)2, PPTS
(ii) H2, 10% Pd/C
O
O
O
H
OR
10
11a, R = BOM
11b, R = H
H2, 10% Pd/C
Swern Oxidation
O
O
O
O
H
12

Fig. 2

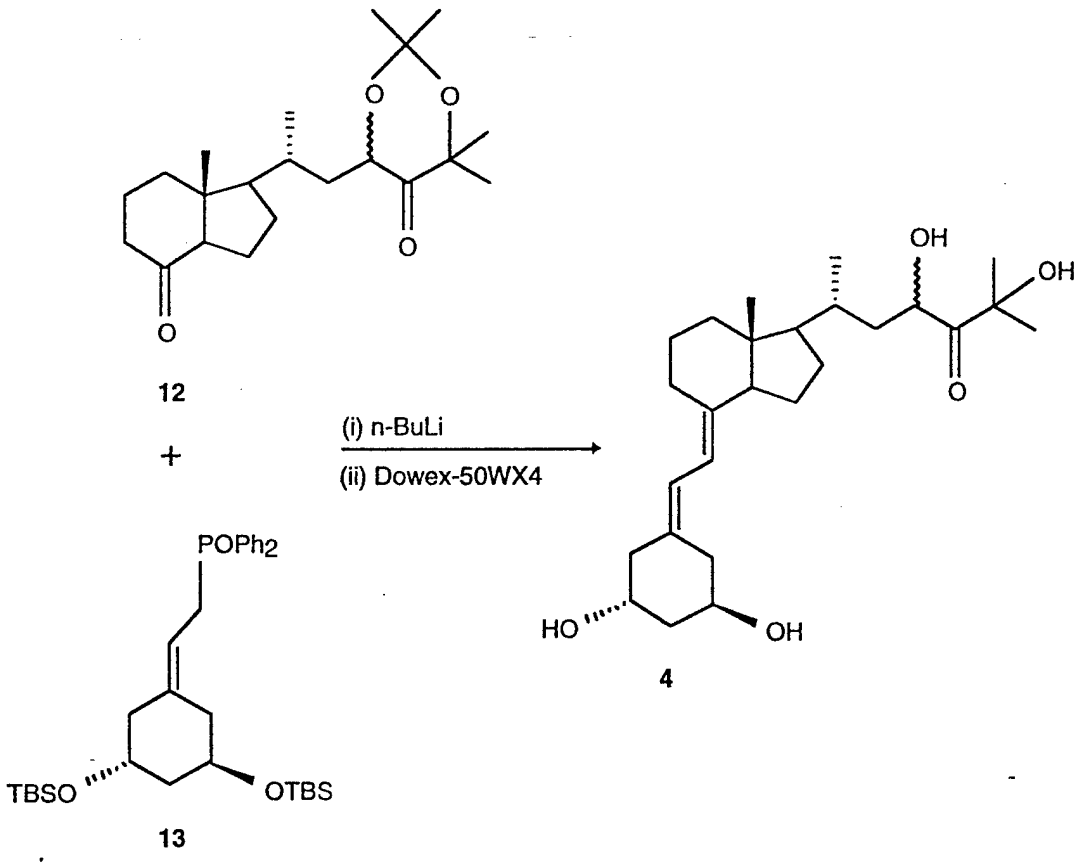


Fig. 3

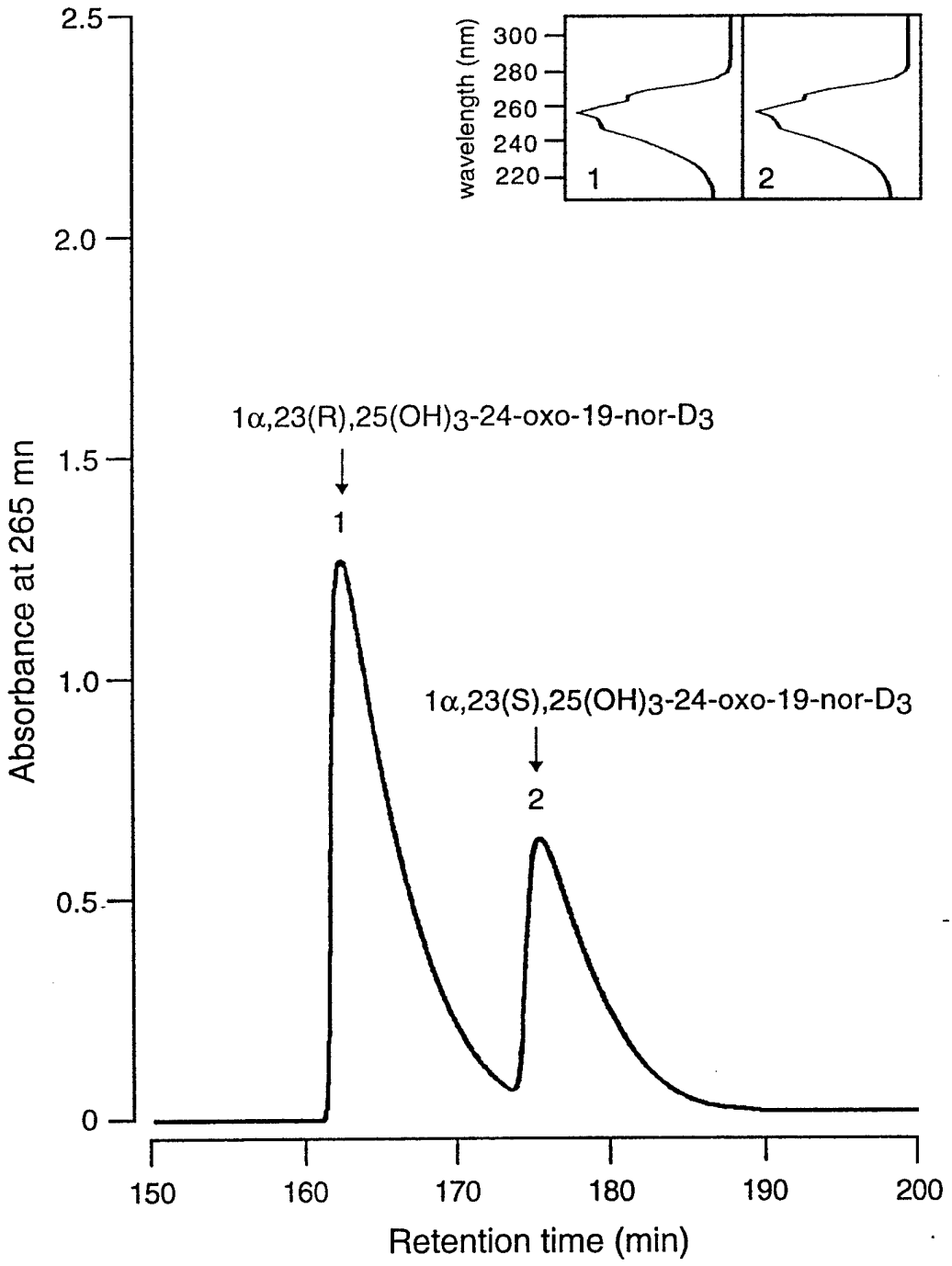

wavelength (nm)
300
280
260
240
220
1
2
2.5
2.0
1.5
1.0
0.5
0
Absorbance at 265 mn
1α,23(R),25(OH)3-24-oxo-19-nor-D3
1
1α,23(S),25(OH)3-24-oxo-19-nor-D3
2
150
160
170
180
190
200
Retention time (min)

Fig. 4

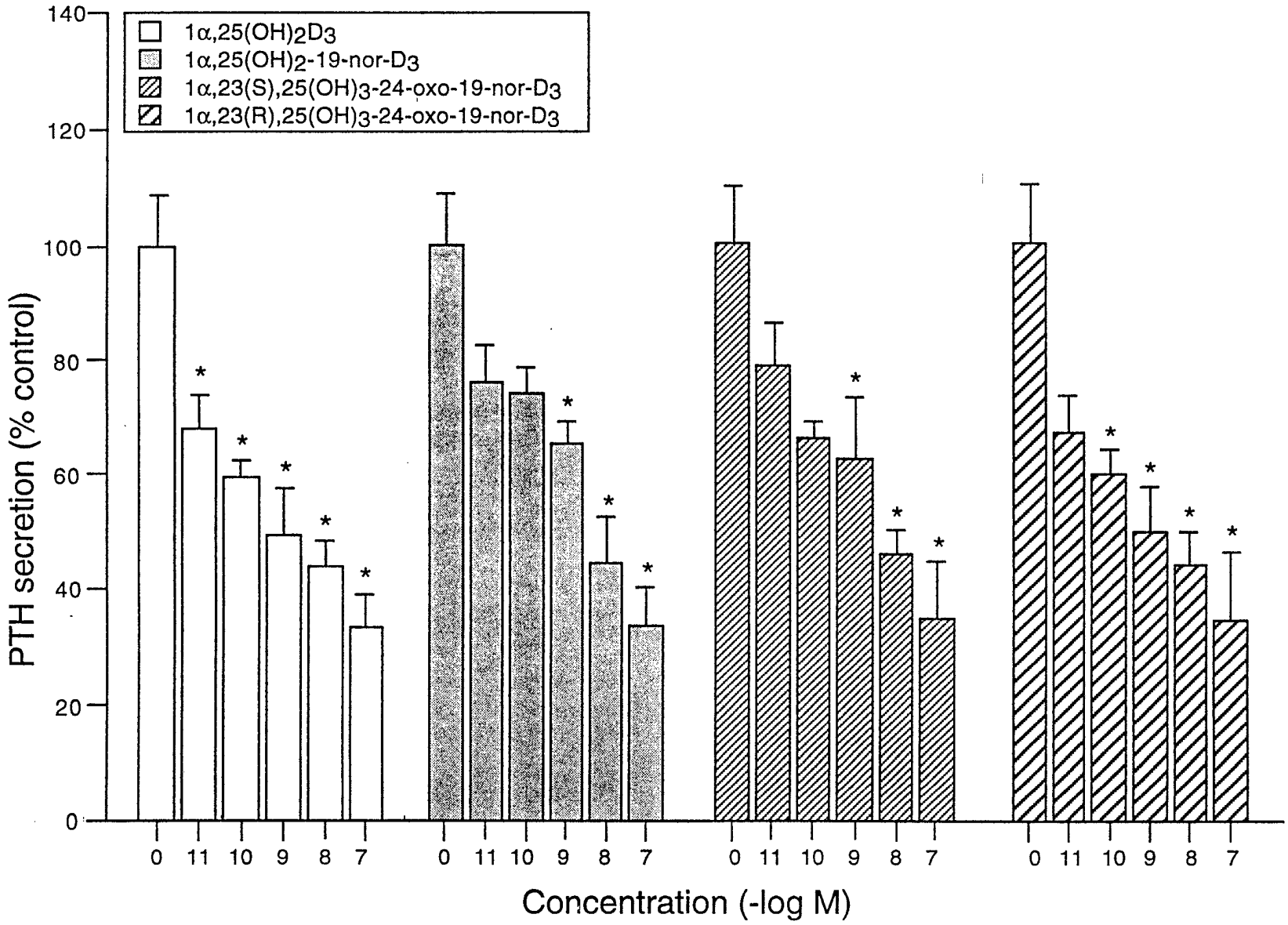

1α,25(OH)2D3
1α,25(OH)2-19-nor-D3
1α,23(S),25(OH)3-24-oxo-19-nor-D3
1α,23(R),25(OH)3-24-oxo-19-nor-D3
PTH secretion (% control)
140
120
100
80
60
40
20
0
0 11 10 9 8 7
0 11 10 9 8 7
0 11 10 9 8 7
0 11 10 9 8 7
Concentration (-log M)

Fig. 5

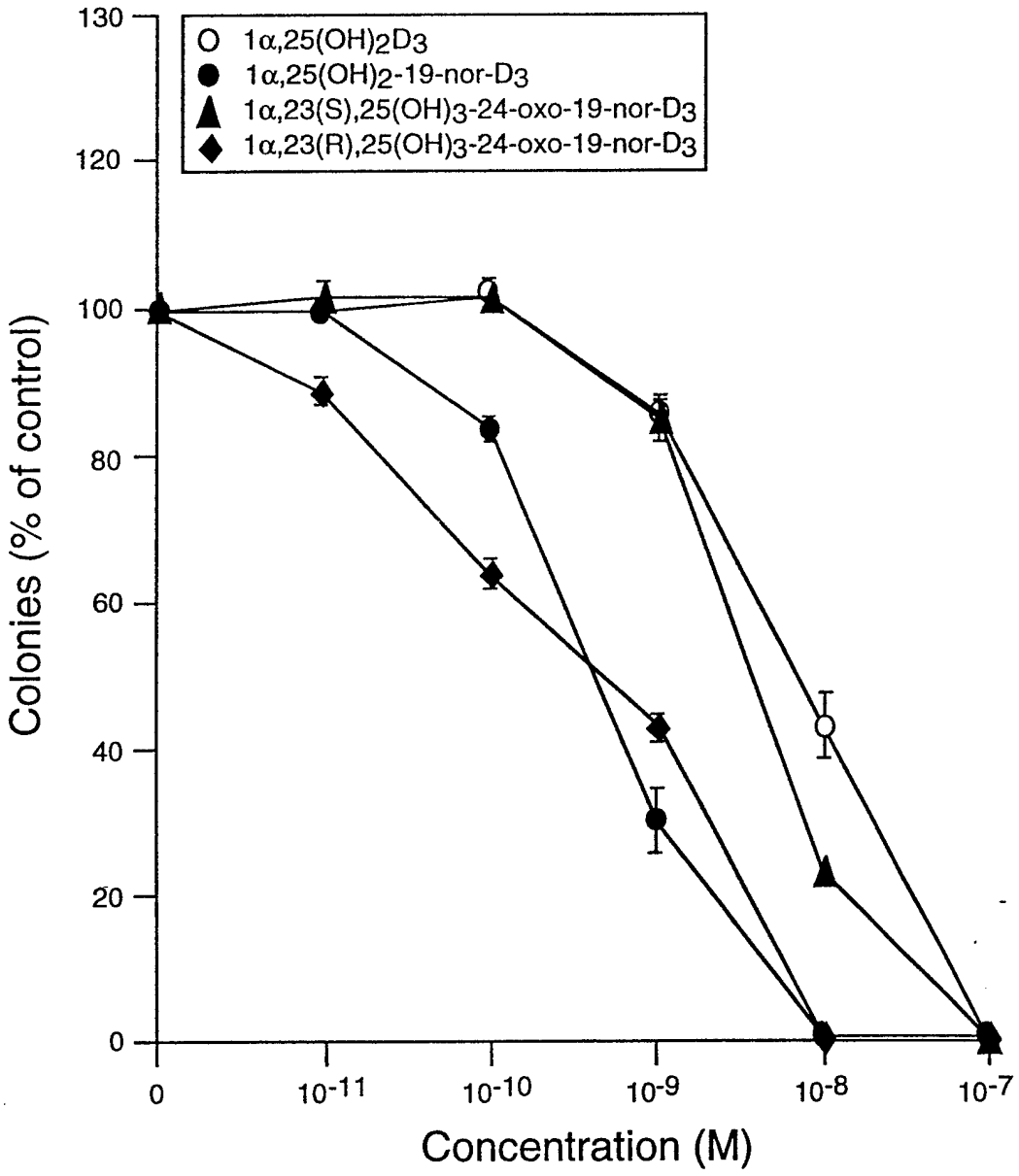

130
120
100
80
60
40
20
0
Colonies (% of control)
0
10-11
10-10
10-9
10-8
10-7
Concentration (M)
O 1α,25(OH)2D3
● 1α,25(OH)2-19-nor-D3
▲ 1α,23(S),25(OH)3-24-oxo-19-nor-D3
◆ 1α,23(R),25(OH)3-24-oxo-19-nor-D3

Fig. 6

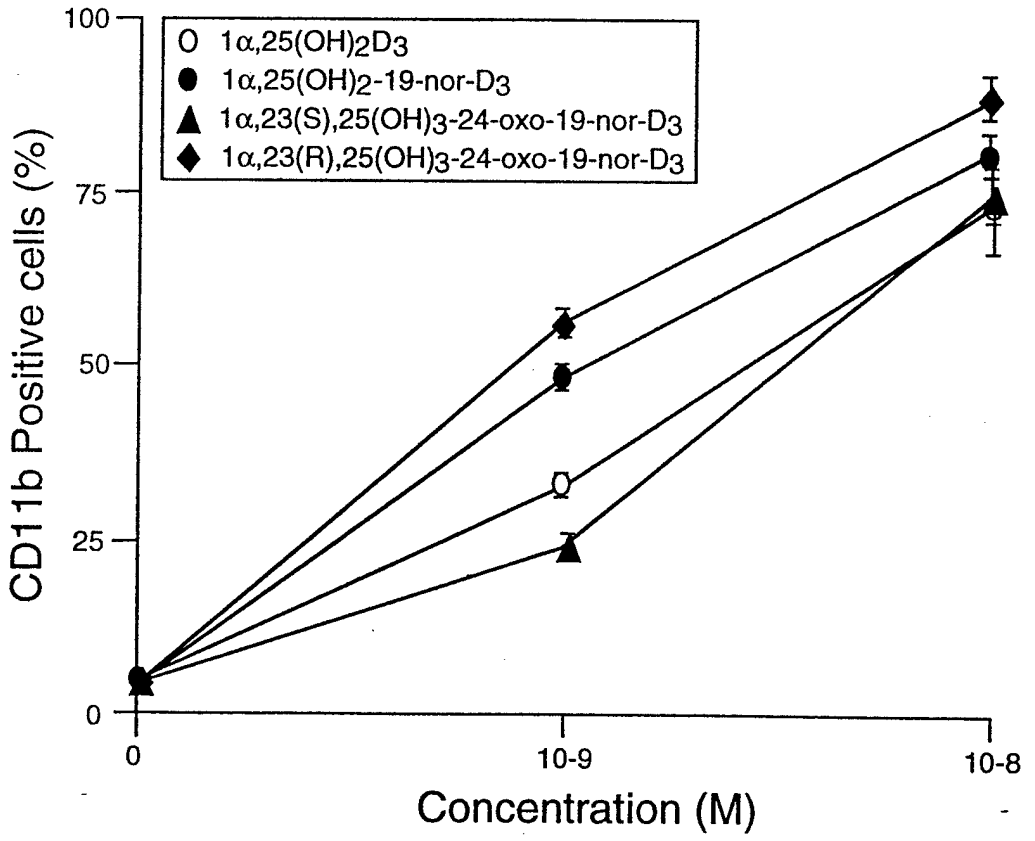

100
75
50
25
0
CD11b Positive cells (%)
1α,25(OH)2D3
1α,25(OH)2-19-nor-D3
1α,23(S),25(OH)3-24-oxo-19-nor-D3
1α,23(R),25(OH)3-24-oxo-19-nor-D3
0
10-9
10-8
Concentration (M)

Fig. 7

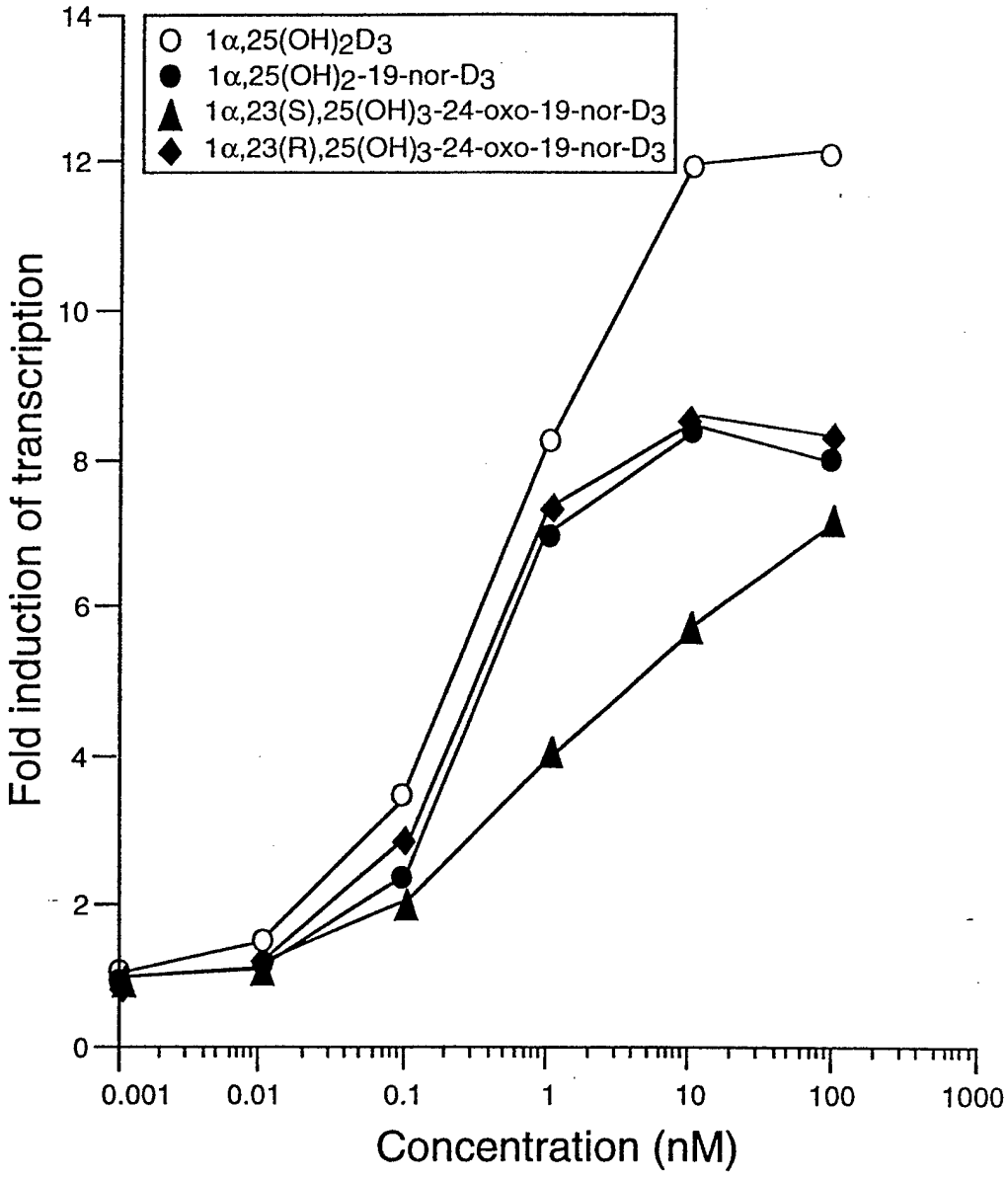

1α,25(OH)2D3
1α,25(OH)2-19-nor-D3
1α,23(S),25(OH)3-24-oxo-19-nor-D3
1α,23(R),25(OH)3-24-oxo-19-nor-D3
Fold induction of transcription
0
2
4
6
8
10
12
14
0.001
0.01
0.1
1
10
100
1000
Concentration (nM)

Fig. 8